Hypoxia-inducible factor-2α-expressing interstitial fibroblasts are the only renal cells that express erythropoietin under hypoxia-inducible factor stabilization

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Erythropoietin (EPO) is the principal regulator of red blood cell production, linking decreased tissue oxygenation to an adequate erythropoietic response. The adult kidney accounts for ~90% of the total EPO response, indicating that its expression is under tight, tissue-specific regulation.1 In this study, the critical importance of hypoxia-inducible transcription factors (HIFs) has been well established. HIFs are heterodimeric transcriptional regulators consisting of distinct oxygen-sensitive α-subunits (such as HIF-1α, HIF-2α, or HIF-3α) and a common, stable β-subunit.2 The oxygen sensitivity of HIFs depends on the action of HIF prolyl hydroxylases (HIF-PHD), which catalyze hydroxylation of proline residues of HIF-α when molecular oxygen is available as a substrate.3 Hydroxylated HIF-α is recognized by the von Hippel Lindau protein to induce proteasomal degradation.4 Under hypoxia, degradation is inhibited and HIF-α can be transferred to the nucleus where it recruits HIF-β and the transcriptional coactivator p300 for its activation.2 In addition to molecular oxygen, HIF-PHD activity depends on the availability of 2-oxoglutarate, ferrous iron, and ascorbate as cofactors. Pharmacological interference with any of these cofactors has been shown to impair oxygen-dependent HIF degradation.5–7 In this study, we used the 2-oxoglutarate analog, FG-4497, to inhibit HIF-PHDs. This compound has been shown by us and others to enhance the nuclear abundance of HIF isoforms, to induce the expression of HIF target genes such as EPO, and to increase its plasma levels without apparent toxicity.8–11

Available data obtained from cell models and transgenic mice have provided evidence for HIF-1α and HIF-2α to contribute to the stimulation of EPO-mRNA expression at least under certain conditions.12–17 In the adult organism, renal EPO expression seems to be preferentially regulated by HIF-2α and not by HIF-1α.14,17–19 Previous work has defined the localization of EPO synthesis in a subset of cortical interstitial fibroblasts, which are characterized by their...
expression of ecto-5'-nucleotidase (5'NU). In agreement with the proposed role of HIF-2α in EPO activation, we have shown earlier that only HIF-2α was expressed in cells of the renal cortical interstitium. In contrast, HIF-1α was distributed in tubular epithelia. This would imply cellular colocalization of HIF-2α and EPO upon adequate activation; to date, however, this has not been shown. In addition, the identity of HIF-2α-expressing cortical interstitial cells remains to be defined. Therefore, we applied FG-4497 in rats as a suitable approach to increase HIF-α abundance and resulting EPO mRNA expression to resolve this issue.

RESULTS
The renal distribution of EPO mRNA signal as well as that of HIF-1α- and HIF-2α-immunoreactive signals was determined in rats treated with FG-4497 or vehicle for 6 h (Figure 1a-d). EPO mRNA signal, as shown by in situ hybridization, was localized to cortical interstitial cells; positive cells were predominantly found in the cortical labyrinth and to a lesser extent in the medullary rays; epithelial and vascular structures were negative (Figure 1b). Control animals did not show reproducible EPO mRNA staining (Figure 1a). The majority of EPO-expressing cells were located in the juxtamedullary (deep) regions of the renal cortex (59 ± 3 cells per mm²), whereas in the superficial cortex, their distribution was more scarce (25 ± 6 cells per mm²; Table 1). HIF immunoreactivity was principally restricted to the cell nuclei. Staining for HIF-1α was localized to epithelial cells, whereas nonepithelial cells were negative (Figure 1c); signals were more frequent in the superficial cortex (952 ± 5 cells per mm²) than in the deep cortex (642 ± 7 cells per mm²; Table 1). Cortical HIF-2α staining in the kidneys of FG-4497-treated rats was localized to interstitial cells and to a number of endothelial and glomerular cells (Figure 1d); interstitial HIF-2α distribution showed no major difference between deep and superficial cortices (123 ± 21 vs 91 ± 12 cells per mm²; Table 1). Kidney samples obtained from control rats were devoid of HIF-α signals (Table 1). Colocalization of EPO mRNA and HIF-α isoforms was evaluated on mirror sections of the kidney (Figure 2a-h). HIF-1α immunoreactivity did not show spatial association with EPO mRNA expression (Figure 2a-d). By contrast, HIF-2α immunoreactivity showed colocalization with EPO mRNA expression in the vast majority of EPO-producing fibroblasts in the superficial and deep regions of the cortex (83 ± 1.4% and 91 ± 0.6%, respectively; Figure 2e-h; Table 1).

To identify the nature of labeled interstitial cells in FG-4497-treated rats, we performed double staining for EPO mRNA and the interstitial fibroblast marker, 5'NU. The resulting frequent coexpression of 5'NU immunoreactive signal and EPO mRNA within the same cells confirmed their nature as fibroblasts, corresponding to previous evidence obtained for this cell type under anemic and hypoxic conditions (Figure 3). In contrast, caveolin-1-immuno-reactive endothelial cells did not display EPO mRNA signal throughout (Figure 4). To characterize HIF-2α-expressing interstitial cells, we performed double labeling for HIF-2α and markers for the cellular constituents of the cortical interstitium. Strong HIF-2α immunoreactivity was found in the nuclei of cells that also expressed the fibroblast marker 5'NU (Figure 5a-c). We further observed HIF-2α staining in a subset of nuclei of RECA-1 (rat endothelial cell antigen-1)-positive endothelial cells (Figure 5d-f). Quantitative assessment of interstitial HIF-2α distribution showed that 74 ± 9.7% of the HIF-2α-immunoreactive nuclei belonged to interstitial fibroblasts and 25 ± 5.6% of them to endothelial cells (Table 2). Major histocompatibility complex II-immuno-reactive dendritic cells and macrophages did not show HIF-2α immunostaining (Figure 5g-i; Table 2).
Table 1 | Quantification of EPO mRNA, HIF-1α, and HIF-2α signals

<table>
<thead>
<tr>
<th></th>
<th>VE IC</th>
<th>FG IC</th>
<th>VE tubule</th>
<th>FG tubule</th>
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<tbody>
<tr>
<td></td>
<td>Deep</td>
<td>Superficial</td>
<td>Deep</td>
<td>Superficial</td>
</tr>
<tr>
<td>EPO (cells per mm² ± s.e.m.)</td>
<td>0 ± 0</td>
<td>59 ± 2.5</td>
<td>25 ± 6</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>HIF-1α (cells per mm² ± s.e.m.)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>642 ± 7.4</td>
</tr>
<tr>
<td>HIF-2α (cells per mm² ± s.e.m.)</td>
<td>0 ± 0</td>
<td>123 ± 21</td>
<td>91 ± 12</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>EPO+HIF-1α (% ± s.e.m.)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>EPO+HIF-2α (% ± s.e.m.)</td>
<td>0 ± 0</td>
<td>91 ± 0.6</td>
<td>83 ± 1.4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>HIF-2α+EPO (% ± s.e.m.)</td>
<td>0 ± 0</td>
<td>51 ± 9.5</td>
<td>28 ± 3.2</td>
<td>0 ± 0</td>
</tr>
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Abbreviations: EPO, erythropoietin; FG, FG-4497; HIF, hypoxia-inducible factor; IC, interstitial cell; VE, vehicle.
Quantitative evaluations of superficial and juxtamedullary (deep) regions of the cortex from VE and FG-treated rats. Signals located in ICs or tubules within deep vs. superficial cortex were evaluated. Upper three lines: Cell counts normalized for sectional area. Lower three lines: Colocalization of EPO and HIF signal as quantified on adjacent mirror sections. Line 5 (EPO+HIF-2α) specifies the percentage of EPO mRNA-positive cells also showing a HIF-2α signal. Line 6 (HIF-2α + EPO) refers to the percentage of HIF-2α-immunoreactive interstitial cells also expressing EPO mRNA.
Data are means ± s.e.m.; n=4 per group.

To validate our model of FG-4497-induced EPO expression, we compared renal cortical EPO mRNA abundance in FG-4497-treated animals with that in animals subjected to isobaric hypoxic hypoxia with a FiO₂ of 0.08. Hypoxia and FG-4497 treatments caused increases in renal cortical EPO mRNA content in a similar range; however, increases were more pronounced in hypoxic rats (26 ± 4-fold of control; P < 0.05) than in FG-4497-treated rats (8.5 ± 1-fold of control; P < 0.05) (Figure 6).

DISCUSSION
Our results, for the first time, show colocalization of EPO mRNA and HIF-2α-immunoreactive signal in interstitial fibroblasts of the renal cortex under the HIF-stabilizing condition of pharmacologically induced HIF-PHD inhibition. This measure was suitable to induce EPO and HIF cellular signals reliably and reproducibly, corresponding to the effects of established, biologically or chemically induced systemic hypoxia. A comparison with hypoxia showed that the obtained increases in EPO mRNA were in a similar range, although more pronounced in hypoxic, than in FG-4497-treated, rats; this shows that the applied dose of FG-4497 (50 mg/kg) caused an effect comparable with hypoxia in the mild to mid-grade range.29 Marked increases in EPO mRNA and both HIF-α isoforms upon FG-4497 treatment were consistent with those of previous studies using HIF-PHD inhibitors.8–11 The distribution of EPO mRNA in FG-4497-treated animals was similar to that observed in animals exposed to systemic hypoxia.20–25 The cellular localization of HIF-α isoforms corresponded to that obtained in animals that had been subjected to hypoxia or to the nonspecific inhibitor of HIF prolyl hydroxylase, cobalt chloride.26–28 Our analysis of serial sections stained for EPO mRNA and HIF-α isoforms showed that the majority of EPO-expressing fibroblasts, which showed highest density in the juxtamedullary portions of the cortical labyrinth, also displayed HIF-2α staining. We could further show that HIF-2α accumulation was also localized in a fraction of capillary endothelial cell nuclei of the peritubular interstitium. Nonetheless, EPO mRNA was principally absent in these cells, which is in agreement with the outcome of a recent transgenic approach.24 Furthermore, EPO and HIF-1α signals were entirely separated, as HIF-1α signals were detected only in tubular epithelia. This is in concordance with previous work,8,26 and in general, earlier data on an epithelial localization of renal EPO synthesis in the adult organism have not received further support from more recent approaches.24,30,31

Our findings are thus consistent with an important role of HIF-2α in the regulation of EPO mRNA expression of the adult kidney. The fibroblast nature of HIF-2α-stained interstitial cells was confirmed by concomitant 5’NU immunostaining and by the absence of major histocompatibility complex II staining, signifying that dendritic cells or macrophages were not involved.32 It is important to note that HIF-2α signals—similar to EPO signals—were only found in a fraction of 5’NU-positive fibroblasts. Furthermore, only a subset of the HIF-2α-positive cells showed concomitant EPO expression, which implies that the cellular accumulation of HIF-2α per se is not sufficient to induce EPO transcription. Therefore, it is likely that further regulatory, as yet unidentified components must act in concert with HIF-2α to produce the typical, restricted distribution pattern of EPO mRNA-expressing fibroblasts. A differential regulation of these components could also be relevant for the HIF-1α-dependent stimulation of EPO transcription under certain conditions.13,15,16 Candidates for these components may be GATA transcription factors, hepatocyte nuclear factor 4, nuclear factor kB, and various protein kinases, which have all been shown to modulate EPO transcription.2,24,33–35 Their involvement in the restriction of EPO in health and disease, as well as during development, must be resolved to advance mechanistic insights into the renal response to hypoxia. HIF-PHD inhibitors may serve as useful tools in this study. Our data provide no compelling evidence that the subset of interstitial fibroblasts coexpressing EPO mRNA and HIF-2α is a cell type of its own; rather, available evidence favors the concept of a highly flexible potential to synthesize EPO that is not necessarily restricted to the innermost renal cortex.23,24,36

In conclusion, we have shown that treatment with the HIF-PHD inhibitor FG-4497 significantly increased abundances in renal HIF-α protein and EPO mRNA levels.
The novel findings in our study are based on cellular colocalization strategies and include (1) the demonstration of HIF-2α expression in cortical interstitial fibroblasts and endothelial cells of the kidney and (2) the documentation that cellular EPO mRNA expression was restricted to cortical interstitial fibroblasts and colocalized with HIF-2α, but not with HIF-1α. These findings supplement functional studies demonstrating a prominent role of HIF-2α in the regulation of renal EPO expression. Our data will further be relevant for clinical trials testing HIF-stabilizing agents for the therapeutic modulation of EPO.

MATERIALS AND METHODS

General

The HIF-PHD inhibitor FG-4497 was provided by FibroGen (San Francisco, CA, USA) and used as described previously. Briefly, FG-4497 was dissolved by sonification in an alkalinized 5% glucose solution (10 mg/dl; pH 8.7) and applied intravenously at a dose of 50 mg/kg body weight. This dose has been previously shown to be well tolerated and to markedly increase plasma EPO levels in mice and Rhesus Macaques within 4–6 h.

Animal models and tissue preparation

Animal studies were conducted in accordance with the German law for animal protection (reference nos G0014/07 and G0189/07 of the Berlin Senate) and NIH policies. Adult male Sprague–Dawley rats (body weight 250 g) were used throughout the study. FG-4497 or vehicle was applied intravenously after anesthesia with isoflurane (inhalation) or intraperitoneal injection of ketamine (100 mg/kg intraperitoneally). Rats were subsequently allowed to regain consciousness for the treatment period of 6 h. After treatment,
the kidneys were either perfusion-fixed with 3% paraformaldehyde through the abdominal aorta (n = 4 per group) or processed for mRNA isolation (n = 5 per group). In a parallel approach, rats were subjected to isobaric hypoxic hypoxia in a sealed hypoxic chamber (FiO2 0.08; 6 h; n = 5 per group). The majority of HIF-2α-immunoreactive cortical interstitial cells also stain positive for 5’NU (arrowheads). Inserts show high-power magnifications of a HIF-2α-positive interstitial fibroblast (c) and a HIF-2α-immunoreactive endothelial cell (f). Arrowheads indicate coexpression of HIF-2α and the respective marker, bars indicate cells expressing the respective marker, and bars indicate cells expressing the respective marker, but not HIF-2α. Original magnification × 400. HIF-2α, hypoxia-inducible factor-2α; MHC II, major histocompatibility complex II; 5’NU, ecto-5’-nucleotidase; RECA-1, rat endothelial cell antigen-1.

Table 2 | Numerical evaluation of HIF-2α- and specific marker-labeled cells

<table>
<thead>
<tr>
<th>Interstitial cells (per × 400 field)</th>
<th>HIF-2α pos. (per × 400 field)</th>
<th>HIF-2α and marker (%)</th>
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<tbody>
<tr>
<td>RECA-1</td>
<td>18 ± 3.3</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>Ecto-5’-nucleotidase</td>
<td>17 ± 1.35</td>
<td>6.9 ± 0.7</td>
</tr>
<tr>
<td>MHC II</td>
<td>17 ± 1.74</td>
<td>6.6 ± 1.5</td>
</tr>
</tbody>
</table>

Abbreviations: DAPI, 4’-6-diamidino-2-phenyl indole; HIF-2α, hypoxia-inducible factor-2α; MHC II, major histocompatibility complex II; pos., positive; RECA-1, rat endothelial cell antigen-1.

Cells were counted per optical field as viewed on double-stained sections from FG-4497-treated tissues. Categories refer to the absolute number of interstitial cells identified by DAPI nuclear stain, number of HIF-2α-positive cells, and percentage of the HIF-2α-positive cells that co-express one of the markers listed. Data are means ± s.e.m.
Colocalization of EPO mRNA and HIF-2α protein was studied on consecutive 3-μm-thick paraffin sections; these were analyzed at their mirror surfaces and the images reoriented electronically. Cell counting was performed in the deep vs superficial halves of the cortex on sections stained for the respective HIF-α isoforms and EPO mRNA. Sections were examined using a Leica DMRB microscope equipped with an interference contrast module (Leica, Wetzlar, Germany). Images were acquired using a SPOT RT 2.3.0 digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and the MetaVue imaging System (Molecular Devices, Downingtown, PA, USA). To optimize the resolution of overview micrographs, two adjacent ×100 pictures of each staining were acquired and subsequently aligned electronically.

Characterization of EPO-expressing cortical interstitial cells was performed by double labeling EPO mRNA-stained sections with antibodies against 5′NU (rabbit anti-CD73; kind gift from Michel LeHir, Zurich, Switzerland) for interstitial fibroblasts, 20,32 and caveolin-1 (Santa Cruz Antibodies, Santa Cruz, CA, USA) for endothelial cells.37 HIF-2α-immunoreactive cortical interstitial cells were characterized by double-staining procedures using mouse monoclonal anti-5′NU (BD Pharmingen, Heidelberg, Germany),38 the endothelial cell marker RECA-1 (ABD Serotec, Oxford, UK),39 or the dendritic cell marker major histocompatibility complex II (Harlan Sera-lab, Belton, UK).35 For double labeling, 6-μm-thick cryostat sections were first stained for RECA-1, 5′NU (rabbit anti-CD73; kind gift from Michel LeHir, Zurich, Switzerland) for interstitial fibroblasts, 20,32 and caveolin-1 (Santa Cruz Antibodies, Santa Cruz, CA, USA) for endothelial cells.37 HIF-2α and subsequently aligned electronically.

Figure 6 | FG-4497 treatment and hypoxia induce renal EPO mRNA expression. Cortical extracts of FG-4497- or hypoxia-treated rats were analyzed by TaqMan real-time PCR for EPO mRNA expression. Data are means ± s.e.m. (n = 5); *P < 0.05. EPO, erythropoietin.

To quantify the extent of colocalization of HIF-2α with the different markers, 10 micrographs of the cortical interstitium, taken at random from FG-4497-treated rats (n = 4), were analyzed (magnification ×400).

Analysis of data

Results were evaluated using routine parametric descriptive statistics. Groups were compared with the unpaired t-test. A probability level of P < 0.05 was accepted as significant. Values are given as means ± s.e.m.

DISCLOSURE

LAF, MA, and SJK are employees of FibroGen Inc. All of the other authors declared no competing interests.

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REFERENCES


