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Reevaluation of erythropoietin production by the nephron

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ABSTRACT

Erythropoietin production has been reported to occur in the peritubular interstitial fibroblasts in the kidney. Since the erythropoietin production in the nephron is controversial, we reevaluated the erythropoietin production in the kidney. We examined mRNA expressions of erythropoietin and HIF PHD2 using high-sensitive in situ hybridization system (ISH) and protein expression of HIF PHD2 using immunohistochemistry in the kidney. We further investigated the mechanism of erythropoietin production by hypoxia *in vitro* using human liver hepatocell (HepG2) and rat intercalated cell line (IN-IC cells). ISH in mice showed mRNA expression of erythropoietin in proximal convoluted tubules (PCTs), distal convoluted tubules (DCTs) and cortical collecting ducts (CCDs) but not in the peritubular cells under normal conditions. Hypoxia induced mRNA expression of erythropoietin largely in peritubular cells and slightly in PCTs, DCTs, and CCDs. Double staining with AQP3 or AE1 indicated that erythropoietin mRNA expresses mainly in β-intercalated or non α/non β-intercalated cells of the collecting ducts. Immunohistochemistry in rat showed the expression of HIF PHD2 in the collecting ducts and peritubular cells and its increase by anemia in peritubular cells. In IN-IC cells, hypoxia increased mRNA expression of erythropoietin, erythropoietin concentration in the medium and protein expression of HIF PHD2. These data suggest that erythropoietin is produced by the cortical nephrons mainly in the intercalated cells, but not in the peritubular cells, in normal hematopoietic condition and by mainly peritubular cells in hypoxia, suggesting the different regulation mechanism between the nephrons and peritubular cells.

1. Introduction

Erythropoietin, which was initially purified from the urine, has a major role in erythrocyte production [1]. Severe anemia stimulates erythropoietin production by 500-fold, and more than 90% of erythropoietin mRNA is found in the kidney of anemic mice [2]. The site of erythropoietin production in the kidney has been a matter of concern. Most studies have reported that interstitial fibroblasts are the site of erythropoietin production in the kidney [3–12]. A few papers have reported that erythropoietin is produced in the proximal or distal tubules [13,14]. Erythropoietin production is stimulated by hypoxia through the activation of hypoxia-inducible transcription factors (HIF) [15–17]. Functional anemia achieved by 0.1% carbon monoxide administration increased HIF1α expression in most of the nephron segments and the interstitial cells, and cobaltous chloride, which mimics hypoxia, increased HIF1α expression in the distal tubules, collecting ducts and interstitial cells [9,18,19]. In contrast, carbon monoxide and cobaltous chloride increased HIF2α expression only in interstitial cells, which suggests that HIF2α-expressing interstitial fibroblasts are the only renal cells that produce erythropoietin under hypoxia [9,18,19]. Further examinations distinguished renal erythropoietin-producing (REP)

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cells among peritubular fibroblasts [3–12]. However, in these reports, mice were subjected to really severe anemia (14–17%, 12%, less than 10%, and less than 15% in hematocrit in Ref. [3, 4, 6, 12], respectively) to show REP cells in the kidney, questioning the physiological role of REP cells for erythropoietin production. There should be a site that produces erythropoietin to maintain erythropoiesis under basal condition. However, no report has shown such site under basal condition so far.

Obara et al. reported that GATA transcription factors play a crucial role in erythropoietin gene expression [12]. They suggested that GATA2 and 3 are mainly expressed in the distal tubules and collecting ducts and that they suppress erythropoietin production at the transcriptional level. These data imply that the distal tubules and collecting ducts are possible sites of erythropoietin production among the nephron segments.

The purpose of our study was to evaluate whether the nephron could produce erythropoietin. We used high sensitivity in situ hybridization (ISH) to detect the mRNAs of erythropoietin and HIF prolyl hydroxylase 2 (PHD2) and immunohistochemistry to detect HIF PHD2. We also used IN-IC cells, which are characterized as intercalated cells to study the mechanisms of erythropoietin production by the nephron [20,21].

2. Materials and methods

2.1. Animals and cell culture

C57BL/6J mice were used for ISH and Sprague Dawley (SD) rats were used for immunohistochemistry. The intercalated cell line (IN-IC cells) was generated as previously described [20,21]. HepG2 cells were purchased from RIKEN (Tsukuba, Japan). Anemia was induced in the SD rats. HepG2 cells were cultured at 37 °C in DMEM with 10% FBS. Because IN-IC cells have the SV40 temperature-sensitive large T antigen, the cells were cultured at 33 °C in DMEM/F12 medium (Life Technologies Japan, Tokyo) with 10% FBS, 10 μg/ml transferrin, 1 μg/ml insulin, 10 ng/ml EGF, 0.5 μg/ml hydrocortisone, 6.5 ng/ml triiodo-tyronine and 1% penicillin/streptomycin. In the hypoxia experiments, IN-IC and HepG2 cells were incubated in 1% O_2 for 24 h. All animal and cell experiments were evaluated and approved by the Committee for Animal Experimentation at the Hyogo College of Medicine (28036, 29014) and Kitasato University Medical Center (25-2).

2.2. In situ hybridization

4 h hypoxia (7% O_2) was applied before the sacrifice of mice [22]. High sensitivity ISH was performed as described previously [23–26]. In brief, total RNA of the mouse kidney (BD Bioscience, Franklin Lakes, USA) was reverse transcribed with an RNA PCR Kit (AMV), ver.3.0 (Takara-Bio, Otsu, Japan), and the cRNA probe for erythropoietin (GenBank accession No. NM_007942) or HIF PHD2 (GenBank accession No. NM_053207) was generated with T7 promoter region-tailed polymerase chain reaction (PCR) primers (Table 1). The hybridized sections were successively treated with 0.1% avidin, 0.01% biotin solution, 0.5% casein/TBS, the horseradish peroxidase (HRP)-conjugated rabbit anti-DIG F (ab′) fragment antibody (DakoCytomation, Glostrup, Denmark), biotinylated tyramide solution and HRP-conjugated streptavidin (DakoCytomation). Staining was visualized with the DAB Liquid System (Bio SB, Santa Barbara, USA) and Mayer's hematoxylin. Precise localization was determined using a double-staining technique with anti-AQP3 (Alomone Labs, Jerusalem Israel) and AE1 (Alpha Diagnostic Intl., San Antonio, USA) antibodies, which are markers of the principal and α intercalated cells, respectively. AEC staining was used for the detection of AQP3 or AE1. Erythropoietin mRNA expression in PCT, medullary thick ascending limbs (MTAL), DCT, intercalated cell (IC), and peritubular cells (peritubular erythropoietin producing cells, PTEC) was quantified as number of dots per cell.

The expression of erythropoietin and HIF PHD2 mRNAs was analyzed using a Zeiss microscope equipped with mercury epifluorescence (Axioplan 2, Carl Zeiss, Jena, Germany) with a digital camera (AxioCam MRc5, Carl Zeiss). Captured images were analyzed using an image analysis system (AxioVision Rel. 4.6, Carl Zeiss).

2.3. Western blot

Western blot analysis was performed as previously described [20,21]. Protein (10–50 μg) from the kidney medulla or IN-IC cells was used for SDS–PAGE. The antibodies against HIF PHD1, 2 and 3 were purchased from Santa Cruz (sc-46024, 271835, and 46030, respectively). Primary and secondary antibodies were used at dilutions of 1000–5000 and 100,000–200,000, respectively. The detection of the bands was performed by Enhanced Chemiluminescence Advance or Prime (GE Healthcare) using LAS-4000 mini (Fuji Film, Japan) in the Research Facilities in Hyogo College of Medicine and in the Kitasato University Medical Center.

2.4. Real time PCR, ELISA and immunohistochemistry

For the hypoxia experiments, IN-IC and HepG2 cells were incubated either at 20 or 1% O_2 for 24 h. Real time PCR of erythropoietin was performed using primers from Applied Biosystems (erythropoietin; Rn01481376_m1, β-actin; Rn00667869). Erythropoietin concentration in the medium or plasma was examined using an ELISA kit (E90028Ra) from USCN Life science (Wuhan, China) and by CLEIA (SRL, Tokyo, Japan), respectively.

SD rats (male, 200–250 g) were made anemic (less than 20% in hematocrit). The kidneys from control and anemic rats were fixed with 1% paraformaldehyde for 60 min at room temperature. After embedding the cells in paraffin, the sections were cut, rinsed and treated with an EDTA solution (pH 9, Nichirei No. 415211, Tokyo, Japan) at 95 °C for 10 min for the antigen retrieval heating. The primary antibody against HIF PHD2 was used at dilutions of 1:50-200. The same doses of mouse IgG were used for the negative control. The secondary antibody was goat-anti mouse IgG. DAB was used for the visualization of the primary antibody. Some of these steps were performed by Applied Medical Research (Osaka, Japan).

Table 1
Primers used to generate a DNA template by RT-PCR for *in vitro* transcription.

Primer	Forward	Reverse
EPO		
Sense probe	5′-CTTAATACGACTCACTATAGGGTCTGCGACAGTCGAGTTCTG-3′	5′-GTGGTATCTGGAGGCGACAT-3′
Antisense probe	5′- TCTGCGACAGTCGAGTTCTG-3′	5′-CTTAATACGACTCACTATAGGGGTGGTATCTGGAGGCGACAT-3′
PHD2		
Sense probe	5′-CTTAATACGACTCACTATAGGGTGGCTCTGGAGTACATCGTG-3′	5′-CGCATCTTCCATCTCCATTT-3′
Antisense probe	5′-TGGCTCTGGAGTACATCGTG-3′	5′-CTTAATACGACTCACTATAGGGCGCATCTTCCATCTCCATTT-3′

RT-PCR, reverse transcription-polymerase chain reaction.

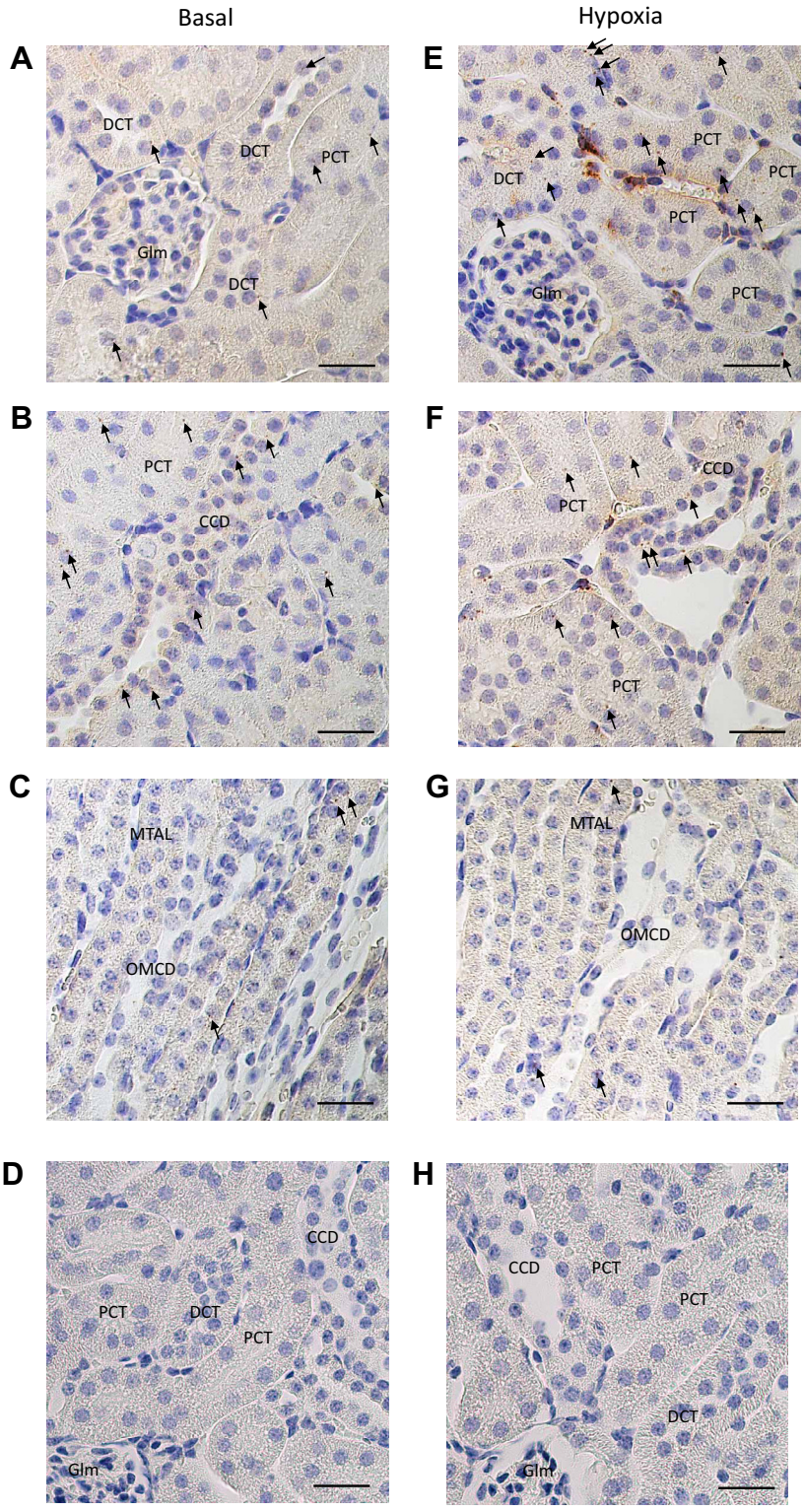

Fig. 1. Erythropoietin mRNA expression in basal and hypoxic states in the kidney. Erythropoietin mRNA expression was observed in the DCTs and CCDs and slightly in the PCTs, OMCDs and MTALs under basal conditions (A–C). Hypoxia increased the expression in the peritubular cells largely and in the DCTs, CCDs and PCTs to some extent but not in OMCDs and MTALs (E–G). Sense probe did not show any signal both in basal and anemic conditions (D and H, respectively). The arrows indicate erythropoietin mRNA in the renal tubules but not in the peritubular cells.

2.5. Statistical analysis

Statistical analyses between or among the samples were performed using Student's *t*-test or ANOVA with multiple comparisons (Dunnett's test), as appropriate. *P* values <0.05 were considered significant, and IBM SPSS (IBM Japan, Tokyo) was used for the analysis.

3. Results

3.1. Erythropoietin mRNA expression in the kidney

Using ISH, erythropoietin mRNAs were probed with antisense and visualized as brown dot-like structures (Fig. 1A–C, E–G) while sense probe did not show any signal (Fig. 1D and H). Erythropoietin mRNA was highly expressed in some cells of the CCDs and slightly expressed in the DCTs and PCTs in control mice (basal condition). There was no erythropoietin mRNA in the interstitial cells under the basal condition (Fig. 1A–C). In the hypoxic mice, the abundance of erythropoietin mRNA was significantly increased in the interstitial cells in the cortex (Fig. 1D and E). The erythropoietin mRNA expression in PCT, DCT, and CCD was also increased. Slight erythropoietin mRNA expression was detected in outer medullary collecting ducts (OMCDs) and medullary thick ascending limbs (MTALs) in basal condition but it was not increased in hypoxic conditions. Double staining with AQP3 (marker for principal cells) or AE1 (marker for α intercalated cells) on the CCDs revealed that erythropoietin mRNA is expressed in the cells that do not express either AQP3 or AE1 (Fig. 2A–D, respectively), suggesting that the cells are β or non-α/non-β intercalated cells. To quantitatively estimate the site of erythropoietin production in the kidney, the dots per cells in PCT, MTAL, DCT, intercalated cell, and peritubular cells were counted. Plasma erythropoietin level was largely increased by 4-h hypoxia (Fig. 2E). Erythropoietin production was observed mainly in the intercalated cells among the nephron but not in peritubular cells in the basal condition (Fig. 2F). Hypoxia stimulated erythropoietin production largely in peritubular cells and slightly in the nephron (Fig. 2F). Dots were observed 47.3 ± 0.3% of intercalated cells in control condition and 19.1 ± 1.3% of peritubular cells in hypoxia. Mean dots were 1.6 ± 0.16 and 4.5 ± 0.3 in intercalated cells and peritubular erythropoietin producing cells (PTEC), respectively, in hypoxia. These data suggest that the intercalated cells are the major sites of erythropoietin production in basal conditions. Therefore, in the following experiments, the intercalated cell line (IN-IC cells) was used to investigate the mechanisms of erythropoietin production.

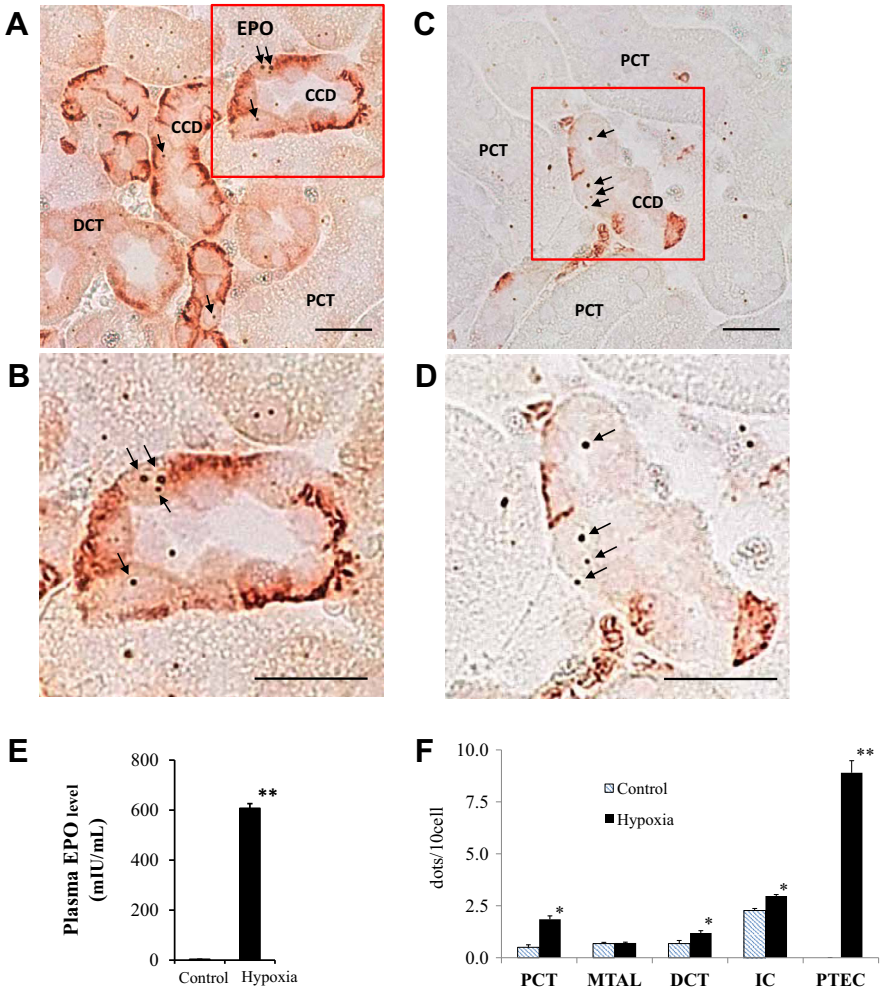

Fig. 2. Localization and quantitative analysis of erythropoietin mRNA expression in the kidney. Erythropoietin mRNA (brown dot) was detected in some cells in the DCTs and CCDs that were not double-stained by anti-AQP3 and anti-AE1 antibodies (A and C, respectively). Enlarged view of the box was showed in the lower column (B and D, respectively). The arrows indicate erythropoietin mRNA in CCDs. Very little erythropoietin mRNA expression was observed in the PCT. (E) Plasma erythropoietin level was largely increased by hypoxia. (F) Erythropoietin mRNA expression was calculated as brown dot per cell in PCT, MTAL, DCT, IC (intercalated cell) and peritubular erythropoietin producing cells (PTEC). Erythropoietin mRNA was detected in PCT, MTAL, DCT and IC in basal condition. Hypoxia induced slight increase in erythropoietin mRNA expression in PCT, DCT and IC and huge increase in PTEC. $^{*}p < 0.05$ vs. basal condition, $^{**}p < 0.01$ vs. basal condition.

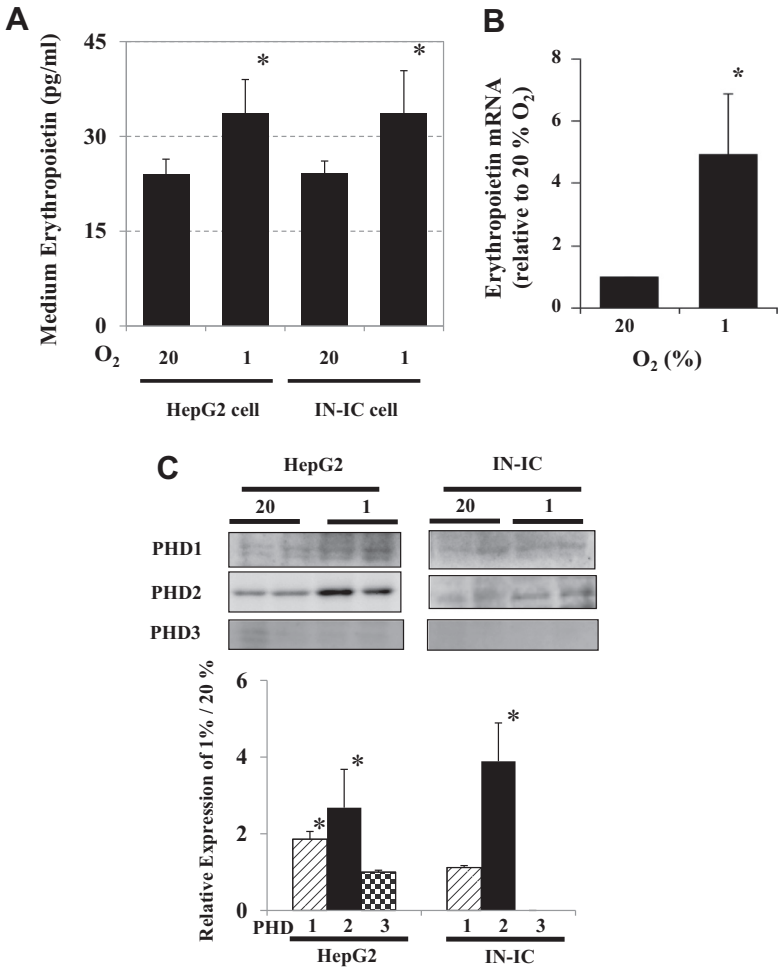

Fig. 3. Erythropoietin expression under basal and hypoxic conditions in HepG2 and IN-IC cells. The erythropoietin level in the medium was increased by hypoxia both in HepG2 and IN-IC cells (A). Hypoxia increased the erythropoietin and erythropoietin receptor mRNA expression levels in IN-IC cells (B). Hypoxia increased PHD1 and 2 but not PHD3 expressions in HepG2 cells and PHD2 but not PHD1 and 3 expressions in IN-IC cells (C). $^{*}p < 0.05$ vs. basal condition.

3.2. Effect of hypoxia in HepG2 cells and IN-IC cells

In HepG2 cells, hypoxia increased the erythropoietin concentration in the medium from 24.0 ± 2.4 to 33.6 ± 5.4 pg/ml (ELISA) ($n = 5$, $p < 0.05$) (Fig. 3A). In IN-IC cells, hypoxia increased the erythropoietin concentration in the medium from 24.2 ± 1.9 to 33.6 ± 6.8 pg/ml ($n = 6$, $p < 0.05$) (Fig. 3A). Hypoxia also increased erythropoietin mRNA expression by 4.94-fold (Fig. 3B).

Hypoxia induced HIF PHD2 expression in both the HepG2 and IN-IC cells and induced HIF PHD1 expression in HepG2 but not IN-IC cells. The expression of HIF PHD3 was not observed in either the HepG2 or IN-IC cells (Fig. 3C).

3.3. HIF PHD2 expression by anemia

Because the increase in HIF PHD2 expression plays a crucial role in the regulation of erythropoietin expression in IN-IC cells, PHD2 expression was examined by ISH (mice) and immunohistochemistry (rats). HIF PHD2 mRNA expression was observed in the PCTs, DCTs and CCDs (Fig. 4A). HIF PHD2 protein expression was observed in DCT and CCD but not in PCT in basal condition (Fig. 4C and E).

Hypoxia stimulated HIF PHD2 mRNA expression in DCTs, CCDs and peritubular cells but not in PCTs (Fig. 4B). Anemia increased the PHD2 protein expression in peritubular cells but not in the DCTs and CCDs (Fig. 4D and F).

4. Discussion

The kidney is a major site of erythropoietin production. For the last 25 years, interstitial fibroblasts, but not the nephron, have been proposed to be a site of erythropoietin production [3–12,15,17]. The primary techniques used for the determination of the erythropoietin production site in the kidney were ISH [3–7] and the study of transgenic mice expressing truncated erythropoietin tagged with GFP [10–12]. However, there is a paper that performed the expression of erythropoietin in microdissected rat nephron using RT-PCR [14]. The administration of cobalt chloride, a mimic of hypoxia, is reported to induce erythropoietin mRNA expression in PCT, cortical thick ascending limbs (CAL) and CCD [14]. We think that because the sensitivity of ISH was not enough 25 years ago, mice need to be subjected to anemic plus hypoxic condition to identify the site of erythropoietin production. Recent reports used the GFP-tagged erythropoietin gene and suggested the possibility that REP cells, such as fibroblasts, are of an extrarenal origin [10,11]. The major problem of these studies was that they involved the induction of very severe anemia, in which the hematocrit is at least less than 17% to observe the interstitial or the REP cells [3,4,6,12]. These findings strongly make us question the role of the REP cells for erythropoietin production in physiological condition. In the present study, we successfully detected erythropoietin mRNA in normal and anemic mice using the high-sensitivity ISH method. Our results indicate that erythropoietin

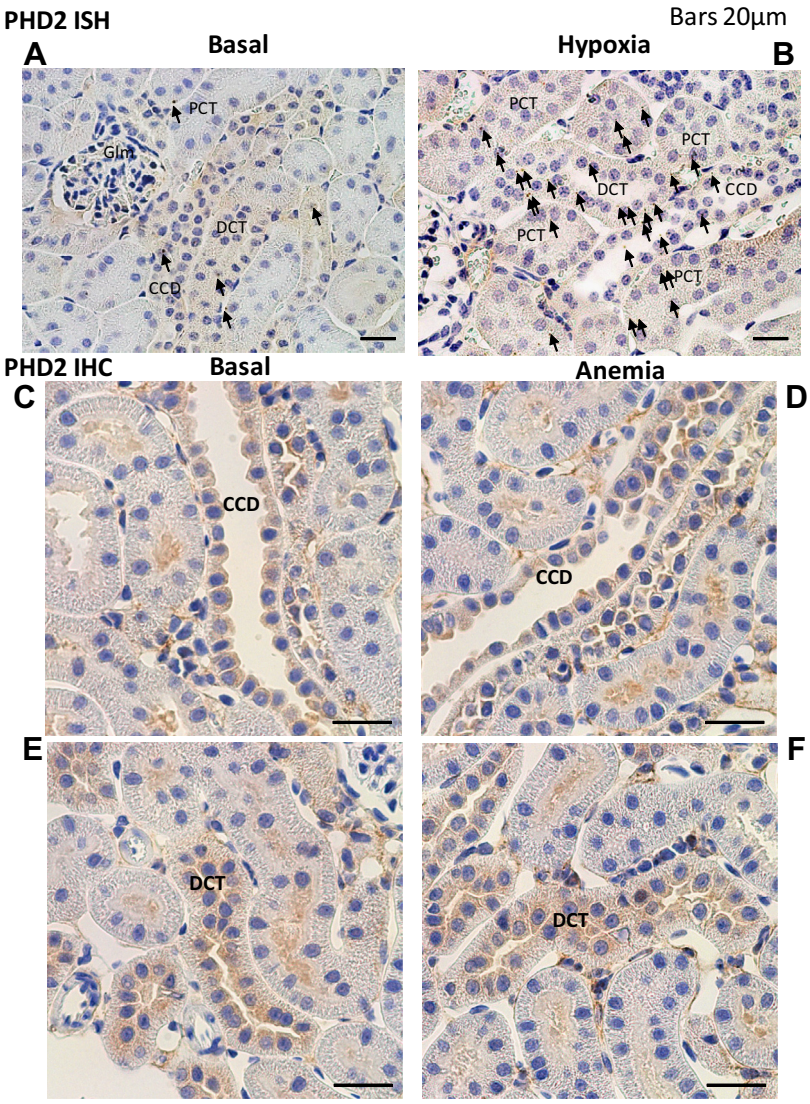

Fig. 4. PHD2 mRNA and protein expression in controls and under anemic conditions. PHD2 mRNA expression was observed in the DCTs, CCDs, and PCTs (A). Anemia increased PHD2 mRNA expression in the DCTs, CCDs and peritubular cells (B). PHD2 protein expression was observed in the DCTs, CCDs but not in PCTs in basal condition (C and E). Anemia stimulated PHD2 protein expression in the peritubular cells but not in DCTs and CCDs (D and F).

production occurs mainly in the intercalated cells of the collecting ducts under normal conditions. Our methods showed strong signal for erythropoietin mRNA in peritubular cells in hypoxic condition, which is consistent with many previous reports. The stimulation of erythropoietin mRNA in the intercalated cells and IN-IC cells by hypoxia is small compared with large increase in peritubular cells in our study and severe anemia-induced 500 fold increase in whole body [2]. We propose that renal tubules contribute to erythropoietin production mainly at basal and anemic or hypoxic conditions slightly.

Not only showing the site of erythropoietin production, we approached a new mechanism for its production in renal tubules. Hypoxia stimulates HIF 1α and 2α expression [15–17]. HIF 2α is thought to be crucial for the stimulation of erythropoietin production in peritubular cells by hypoxia [9,18,19]. In contrast to HIF2α expression in peritubular cells, renal tubules dominantly express HIF1α. Hypoxia is also known to stimulate HIF PHD 1, 2 and 3, in which HIF PHD2 is responsible for the degradation of HIF2α. In the present study, we showed that hypoxia increased HIF PHD2 expression in HepG2 and IN-IC cells. ISH and immunohistochemistry analysis revealed the PHD2 expression in DCTs, CCDs, and peritubular cells, although the increase in protein expression by anemia in DCT and CCD was not significant. Considering the different pattern of erythropoietin expression between in renal tubules and peritubular cells in basal and hypoxia conditions, there could be a different regulation mechanism of erythropoietin production in renal tubules from peritubular cells.

In conclusion, our data showed that erythropoietin is constitutively produced by the cortical nephrons but not in the peritubular cells in normal hematopoietic condition. Severe anemia stimulates its production mainly in peritubular cells and slightly in the nephron. Regulation mechanism of erythropoietin production by the nephron is different from that by peritubular fibroblasts. Erythropoietin production by the peritubular fibroblasts occurs only in severe anemia and may not be physiologically important.

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References

[1] T. Miyake, C.K. Kung, E. Goldwasser, Purification of human erythropoietin, J. Biol. Chem. 252 (1977) 5558–5564.
[2] M.J. Koury, M.C. Bondurant, S.E. Graber, S.T. Sawyer, Erythropoietin messenger RNA levels in developing mice and transfer of 125I-erythropoietin by the placenta, J. Clin. Invest. 82 (1988) 154–159.
[3] S.T. Koury, M.C. Bondurant, M.J. Koury, Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization, Blood 71 (1988) 524–527.
[4] S. Bachmann, M. Le Hir, K.U. Eckardt, Co-localization of erythropoietin mRNA and ecto-5'-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin, J. Histochem. Cytochem. 41 (1993) 335–341.
[5] G.L. Semenza, S.T. Koury, M.K. Nejfelt, J.D. Gearhart, S.E. Antonarakis, Cell-type-specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 8725–8729.
[6] C. Lacombe, J.L. Da Silva, P. Bruneval, J.G. Fournier, F. Wendling, et al., Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney, J. Clin. Invest. 81 (1988) 620–623.
[7] S.J. Schuster, S.T. Koury, M. Bohrer, S. Salceda, J. Caro, Cellular sites of extrarenal and renal erythropoietin production in anaemic rats, Br. J. Haematol. 81 (1992) 153–159.
[8] P.H. Maxwell, M.K. Osmond, C.W. Pugh, A. Heryet, L.G. Nicholls, et al., Identification of the renal erythropoietin-producing cells using transgenic mice, Kidney Int. 44 (1993) 1149–1162.
[9] A. Paliege, C. Rosenberger, A. Bondke, L. Sciesielski, A. Shina, et al., Hypoxia-inducible factor-2alpha-expressing interstitial fibroblasts are the only renal cells that express erythropoietin under hypoxia-inducible factor stabilization, Kidney Int. 77 (2010) 312–318.
[10] N. Asada, M. Takase, J. Nakamura, A. Oguchi, M. Asada, et al., Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosisand renal anemia in mice, J. Clin. Invest. 121 (2011) 3981–3990.
[11] X. Pan, N. Suzuki, I. Hirano, S. Yamazaki, N. Minegishi, et al., Isolation and characterization of renal erythropoietin-producing cells from genetically produced anemia mice, PLoS One 6 (2010) e25839.
[12] N. Obara, N. Suzuki, K. Kim, T. Nagasawa, S. Imagawa, et al., Repression via the GATA box is essential for tissue-specific erythropoietin gene expression, Blood 111 (2008) 5223–5232.
[13] I. Beirão, L. Moreira, T. Barandela, L. Lobato, P. Silva, et al., Erythropoietin production by distal nephron in normal and familial amyloidotic adult human kidneys, Clin. Nephrol. 74 (2010) 327–335.
[14] S.K. Mujais, N. Beru, T.N. Pullman, E. Goldwasser, Erythropoietin is produced by tubular cells of the rat kidney, Cell Biochem. Biophys. 30 (1999) 153–166.
[15] W. Jelkmann, Regulation of erythropoietin production, J. Physiol. 589 (2011) 1251–1258.
[16] F.S. Lee, M.J. Percy, The HIF pathway and erythrocytosis, Annu. Rev. Pathol. 6 (2011) 165–192.
[17] G.L. Semenza, Involvement of oxygen-sensing pathways in physiologic and pathologic erythropoiesis, Blood 114 (2009) 2015–2019.
[18] S. Mathia, A. Paliege, R. Koesters, H. Peters, H.H. Neumayer, et al., Action of hypoxia-inducible factor in liver and kidney from mice with Pax8-rtTA-based deletion of von Hippel-Lindau protein, Acta Physiol. (Oxf) 207 (2013) 565–576.
[19] C. Rosenberger, S. Mandriota, J.S. Jürgensen, M.S. Wiesener, J.H. Hörstrup, et al., Expression of hypoxia-inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys, J. Am. Soc. Nephrol. 13 (2002) 1721–1732.
[20] Y. Izumi, K. Hori, Y. Nakayama, M. Kimura, Y. Hasuike, et al., Aldosterone requires vasopressin v1a receptors on intercalated cells to mediate acid-base homeostasis, J. Am. Soc. Nephrol. 22 (2011) 673–680.
[21] K. Hori, T. Nagai, Y. Izumi, M. Kimura, Y. Hasuike, et al., Vasopressin V1a receptor is required for nucleocytoplasmic transport of mineralocorticoid receptor, Am. J. Physiol. Renal Physiol. 303 (2012) F1080–F1088.
[22] T. Kobayashi, H. Yanase, T. Iwanaga, R. Sasaki, M. Nagao, Epididymis is a novel site of erythropoietin production in mouse reproductive organs, Biochem. Biophys. Res. Commun. 296 (2002) 145–151.
[23] H.M. Kerstens, P.J. Poddighe, A.G. Hanselaar, A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine, J. Histochem. Cytochem. 43 (1995) 347–352.
[24] H. Yang, I.B. Wanner, S.D. Roper, N. Chaudhari, An optimized method for in situ hybridization with signal amplification that allows the detection of rare mRNAs, J. Histochem. Cytochem. 47 (1999) 431–446.
[25] T. Suzuki, Y. Kadoya, Y. Sato, K. Handa, T. Takahashi, et al., The expression of pancreatic endocrine markers in centroacinar cells of the normal and regenerating rat pancreas: their possible transformation to endocrine cells, Arch. Histol. Cytol. 66 (2003) 347–358.
[26] Y. Yasuoka, M. Kobayashi, Y. Sato, M. Zhou, H. Abe, et al., The intercalated cells of the mouse kidney $OMCD_{is}$ are the target of the vasopressin V1a receptor axis for urinary acidification, Clin. Exp. Nephrol. 17 (2013) 783–792.