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Expression of EPO and related factors in the liver and kidney of plain and Tibetan sheep

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Summary. Erythropoietin (EPO), hypoxia-inducible factor-1α (HIF-1α), hypoxia-inducible factor-2α (HIF-2α), and vascular endothelial growth factor (VEGF) are key factors in the regulation of hypoxia, and can transcriptionally activate multiple genes under hypoxic conditions, thereby initiating large hypoxic stress in the network. The liver and kidneys are important metabolic organs of the body. We assessed the expression of EPO, HIF-1α, HIF-2α, and VEGF in liver and kidney tissues of plain and Tibetan sheep using hematoxylin and eosin staining, immunohistochemistry, and RT-qPCR. The results showed that EPO, HIF-1α, HIF-2α, and VEGF were expressed in tubular epithelial cells, collecting duct epithelial cells, mural epithelial cells, and the glomerular cytoplasm of Tibetan sheep, and their expression was significantly higher in Tibetan sheep than in plain sheep (P<0.05). EPO, HIF-1α, HIF-2α, and VEGF are expressed in hepatocytes, interlobular venous endothelial cells, and interlobular bile duct epithelial cells. In plain sheep, positive signals for EPO, HIF-1α, HIF-2α, and VEGF were localized mainly in interlobular venous endothelial cells, whereas VEGF and HIF-2α were negatively expressed in interlobular bile duct epithelial cells and positively expressed in EPO and HIF-1α. The differences in EPO, HIF-1α, and HIF-2α in Tibetan sheep were significantly higher than those in plain sheep (P<0.001). In the liver and kidney tissues of Tibetan sheep, EPO was associated with HIF-1α, HIF-2α, and VEGF (P<0.05). RT-qPCR results showed that EPO was not expressed, and HIF-1α, HIF-2α, and VEGF were expressed (P<0.05). The results showed that the expression of EPO, HIF-1α, HIF-2α, and VEGF in the kidney and liver of Tibetan sheep was higher than that in of plain sheep. Therefore, EPO, HIF-1α, HIF-2α, and VEGF may be involved in the adaptive response of plateau animals, which provides theoretical clarity to further explore the adaptive mechanism of plateau hypoxia in Tibetan sheep.

Key words: Tibetan sheep, Plain sheep, Liver, Kidney, Hypoxia adaptation

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Introduction

Oxygen is an important substrate for aerobic organisms to maintain their metabolism and physiological functions, and low oxygen concentration is an important ecological factor that affects the survival of animals in a plateau environment (Majmundar et al., 2010). The Tibetan sheep is one of the three primitive sheep strains in China and lives mainly in Qinghai-Tibet, which has the highest altitude in the world. Animal survival and reproduction on the Tibetan Plateau are hampered by a severe environment of low temperatures, limited oxygen, and high ultraviolet radiation. Tibetan sheep, one of the largest indigenous mammals on the Tibetan Plateau, have evolved anatomical morphology, physiology, and behavioral adaptations to their specific ecological environment through long-term natural selection (Liu et al., 2020). The liver and kidneys of animals are organs involved in a variety of physiological functions such as detoxification, metabolism, and excretion; therefore, they obtain more energy from oxygen to support vital activities in the body and are more susceptible to hypoxic regulation.

Erythropoietin (EPO), a glycoprotein hormone belonging to the type I cytokine superfamily, is mainly produced by capillary endothelial cells around the renal tubules in the renal cortex and outer medulla, and promotes the proliferation and differentiation of

erythroid progenitor cells, and mobilizes hematopoietic stem cells (Madonna et al., 2009). Originally defined as a hematopoietic growth factor regulated by hypoxia, EPO is regulated by HIF under normoxia and hypoxia and acts by binding to specific erythropoietin receptors (EPORs) on cell membranes as a result of hypoxia or plateau exposure that decreases tissue oxygen partial pressure and stimulates its release. The relationship between hypoxia, and EPO, and erythrocytes is an important key to studying HIF and its pathways.

HIF is a heterodimer composed made up of an unstable alpha subunit and a stable beta subunit that plays a crucial role function in the internal environment of the body in the absence of oxygen (Pawlus and Hu, 2013). HIF-1α, HIF-2α, and HIF-3 are three members of the HIF-α family. HIF-1α is being encoded by the HIF-1α gene on chromosome 14 (14q23.2) and is a polypeptide with 11 amino acids (Johnson et al., 1991). The half-life of HIF-1α is very short under normoxic conditions and requires multiple factors to regulate HIF hydroxylase activity (Bishop and Ratcliffe, 2014), whereas hypoxia can improve HIF-1 protein stability by inducing HIF-1a mRNA transcription, which promotes HIF target gene transcription (Kudo et al., 2007), such as glucose transporter protein 1 (GLUT1), vascular endothe1ial growth factor (VEGF), and EPO.

HIF-2 was first cloned into endothelial cells in 1997 by Tian et al. It is also known as endothelial PAS domain protein 1 (EPAS1), which in humans is encoded by the HIF-2α gene on chromosome 2 and is a polypeptide containing 870 amino acids. Hypoxia is the predominant factor that regulates HIF-2α protein expression. These two α subunits may be regulatory yet complementary players in the adaptive response to tissue hypoxia (Patel and Simon, 2008) and play important roles in numerous physiological processes, such as energy generation, angiogenesis, tumorigenesis, and hematopoiesis in the body (Gu et al., 1998).

VEGF are major proteins that act on vascular endothelial cells (ECs) and are widely present in the free form in various tissues and organs of the body, with their specific receptors forming the VEGF/VEGFR system, which together participate in changes in permeability, growth, survival, and proliferation of EC, regenerating neurons, and revascularization in the physiological or pathological state of the vascular endothelial fine run (Gallicchio et al., 2005). According to previous research, both HIF-1α and HIF-2α activate the transcription of VGEF genes and increase the stability of VGEF mRNA, promoting neovascularization and better tissue oxygen and nutrient supply (Zimmer et al., 2004).

Most studies have shown that EPO, HIF-1α, HIF-2α, and VEGF are the primary transcriptional regulators of hypoxia; however, how do the effects of hypoxia on the kidney and liver differ from those of highland yak cattle and low-altitude sheep has not been reported. The present study focused on the expression of EPO, HIF-1α, HIF-2α and VEGF in the liver and kidney tissues of plain and Tibetan sheep, which is highly innovative and valuable for investigation, aiming to provide a theoretical basis for the mechanism of hypoxia in highland animals by EPO, HIF-1α, HIF-2α, and VEGF, and provide a research basis for the adaptive level of hypoxia in highlands.

Materials and methods

Materials

Liver and kidney tissues were collected from Tibetan sheep (male, 3-5 years old, n=6) in Linxia City (Gansu Province, China, approximately 3200 m above sea level, approximately 70% of the oxygen in the plains, -10°C) and from plain sheep (male, 3-5 years old, n=6) in Lanzhou City (Gansu Province, China, approximately 1200 m above sea level, oxygen content 20%, -4°C). The animals were euthanized with sodium phenobarbital (200 mg/kg, intravenously) at a local abattoir according to with local regulations. Immediately after euthanasia, samples were collected and preserved in 4% paraformaldehyde for tissue fixation. All Tibetan and plain sheep were considered clinically healthy based on the results of physical examination and serum biochemical tests. The Animal Ethics Committee of the Northwest University for Nationalities also approved this study.

Hematoxylin and eosin staining detection

The liver and kidney tissues of Tibetan sheep were dehydrated in different gradients of alcohol, made transparent in xylene, embedded in wax, and sectioned continuously at 5 μm to make paraffin sections.

The sections were dewaxed in gradient alcohol, stained with hematoxylin for 2 min, washed twice in running water for 5 min, and fractionated twice with 1% hydrochloric acid for 2 s each time. The sections were then rinsed with running water for 10 min before being stained with eosin for 1 min, dehydrated in gradient alcohol, and made transparent in xylene solution for 20 min before being sealed with neutral resin.

Immunohistochemistry detection

After dewaxing in water, the sections were exposed to antigen repair using the citrate buffer-microwave thermal repair procedure: mouse anti-HIF-1α (1:200 dilution, bs-20398; RBioss, China), mouse anti-HIF-2α (1:200 dilution, bs-1447R; RBioss), and rabbit anti-VEGF (1:200 dilution, bs-1665R; RBioss) were added dropwise to the sections and incubated overnight at 4°C according to the manufacturer instructions. The sections were washed with Phosphate buffer solution (PBS, pH=7.2) three times and then incubated with secondary antibody at 37°C for 15 min, followed by incubation with tertiary antibody 37°C for 15 min. The sections were washed after applying the Diaminobenzidine (DAB) chromogenic solution for color development and

finally restained and sealed with neutral resin. PBS was used as the primary antibody negative control, and the other operations were the same as those in the experimental group. Before the experiment, we needed to perform the preparation work, configure the PBS and citrate buffers according to the ratio, preheat the tissue sections by spreading and baking in an oven to thaw the wax (58°C, 2h), freeze the antibodies at 4°C in the refrigerator, and thaw them in time before use.

Extraction and reverse transcription of total tissue RNA

The livers and kidneys of Tibetan and plain sheep were ground rapidly in liquid nitrogen, total RNA was extracted using TRIzol Reagent (Invitrogen, USA), and the concentration and purity of the RNA were measured using a UV spectrophotometer (Biospec-nan0, Japan). First-strand cDNA was synthesized using the FastKing RT Kit (with gDNase) and FastKing cDNA First-Strand Synthesis Kit (TIANGEN BIOTECH, Beijing, China), and then stored at -20°C.

Quantitative real-time polymerase chain reaction ((RT-qPCR)

Primers were used to detect the gene sequences of EPO, HIF-1α, HIF-2α, and VEGF (Table 1). The fluorescent quantitative PCR amplicon reaction system was 20 μL:10 μL SYBR Gene Mix, 8μL ddH2O, 1 μL cDNA upstream and downstream primers each 5 μL. The following reaction conditions was used: 95°C for 4 min, 95°C 30s, 60°C 30 s, 72°C 10 s, and 72°C for 5 min; repeated for 40 cycles. Three replicate wells were used up for each gene.

Statistical analysis

Images were captured using a light microscope (CX31; Olympus, Tokyo, Japan). Image-Pro Plus (version 6.0; Media Cybernetics, Inc., Bethesda, MD, USA) was used to quantify the results of protein level, and the experimental data of RT-qPCR were compiled using Excel 2010. The cycle threshold (Ct) of each sample was used to calculate the relative expression of the target gene by the $2^{-\Delta\Delta Ct}$ method.

All data were expressed as mean ± standard error (SEM), and SPSS 20.0 was used to check for normality and chi-square tests. Comparative analysis was done using the one-way (ANOVA) Duncan technique, and statistical significance was expressed as *, *P<0.05, **P<0.01, and ***P<0.001.

Results

Histological observation

The results showed that the livers and kidney tissues of Tibetan and plain sheep were histologically intact (Fig. 1). The urinary ducts of Tibetan and plain sheep consisted of glomeruli, tubules, and collecting ducts. The parenchyma mainly consisted of renal tubules and tubules, of which the renal tubules were omitted spherical, and from the inside to the outside were the glomerulus and capsule. The glomerulus consisted of arteriolar capillaries, which were coiled into a spherical shape. The cupular structure of the capsule was surrounded by the central glomerulus, and the outer epithelial cells of the capsule were surrounded by a single layer of flattened epithelial cells.

Renal tubules mainly consist of proximal and distal tubules in the cortex and proximal and distal rectal tubules in the medulla; and the periphery of the tubules is rich in collagen fibers.

The hepatic tissue is composed of hepatic lobules and hilar regions. The hepatic lobules include hepatocyte cords, central veins, bile ducts, and hepatic sinusoids. The hepatocytes were well-defined and polygonal with large nuclei and distinct nucleoli. The portal area consists of three companion ducts: the interlobular vein, the interlobular artery, and the interlobular biliary duct. The interlobular vein was a branch of the portal vein, with a large and irregular lumen and scattered smooth muscle fibers outside the endothelium. The interlobular artery was a branch of the hepatic artery, with a circular smooth muscle layer outside the endothelium. The interlobular bile duct was a branch of the hepatic duct, with a single layer of cuboidal epithelium in the duct wall, neatly arranged cells, round nucleus, and lightly stained cytoplasm.

Immunohistochemical localization

Antibody staining for EPO, HIF-1α, HIF-2α, and VEGF was observed in both kidney and liver tissues of Tibetan and plain sheep. Both renal and hepatic expression levels were higher in Tibetan sheep than in plain sheep.

In both Tibetan and plain sheep tissues,

Table 1. Details of the primer sequences used for RT-qPCR.

	forward	reverse
EPO	5'-GTCCCAGTGCCTAAGTGGAA-3'	5'-CAGCAGGTTGTGGTTT-3'
HIF-1α	5'-GACCCTGCACTCAACCAAGA-3	5'-TGGGACTGTTAGGCTCAGGT-3'
VEGF	5'-TGGGTACATTGGAGCCTTGC-3	5'-ACCACTTCGTGGGGTTTCTG-3
HIF-2α	5'-ACCGGGCAAGTGAGAGTC-3	5'-GATGTCCATGTGGGATGGGT-3

immunoreactivity of EPO, HIF-1α, HIF-2α, and VEGF was mainly observed in the glomeruli and tubules (Fig. 1A-O). Immune expression was found in tubular epithelial cells, collecting duct epithelial cells, mural epithelial cells, and glomerular cytoplasm, with the strongest cytoplasmic immunoreactivity in proximal tubular epithelial cells and distal epithelial cells; collecting duct epithelial cells was higher (Fig. 2A,I) and HIF-2α expression was lower (Fig. 2D,L). Staining expression in pain sheep was approximately the same as that in Tibetan sheep, and both had glomerular and tubular expressions (Fig. 5B,F,G). However, the strong positive expression signals for EPO, HIF-1α, HIF-2α, and VEGF were significantly higher in Tibetan sheep than in plain sheep, and the difference was significant.

Positive expression of EPO, HIF-1α, HIF-2α, and VEGF was detected in the livers of both Tibetan and plain sheep in the positive control group (Fig. 3A-O). In Tibetan sheep, EPO, HIF-1α, HIF-2α and VEGF expressions were observed in the portal region (interlobular arteries, interlobular veins, and interlobular bile ducts), where immunoreactivity was observed in hepatocytes, interlobular vein endothelial cells, and interlobular bile duct epithelial cells, with more cytoplasmic than nuclear immunoreactivity observed in hepatocytes. Positive signals for EPO, HIF-1α, HIF-2α, and VEGF were also found in the livers of plain sheep, and EPO was highly positive in plain sheep interlobular vein endothelial cells and interlobular bile duct epithelial cells of plain sheep. Although EPO, HIF-1α, HIF-2α, and VEGF were widely expressed in hepatocytes, their expression was weak and mainly localized in interlobular venous endothelial cells. VEGF and HIF-2α were negatively expressed in interlobular bile duct epithelial cells, while they were positively expressed in EPO and HIF-1α.

The mean integrated optical density values (IOD) results (Fig. 5I-L) showed that the relative expression of EPO, HIF-1α, HIF-2α, and VEGF in the kidney of Tibetan sheep was significantly higher than that of plain sheep, with a significant difference ($P<0.05$) (Fig. 5E-H), The expression in the liver of Tibetan sheep was also higher than that of plain sheep, with a highly significant difference ($P<0.001$) (Fig. 5A-D).

Discussion

Hypoxic adaptation is the collection of protective mechanisms developed by the body to maintain essential life activities when oxygen acquisition and delivery are impaired by hypoxic environments, high levels of hypoxia, or disease. Four genes (EPO, HIF-1α, HIF-2α, and VEGF) have been identified as important transcription factors regulating hypoxic signaling, which has been previously highlighted in the literature. In this study, the expression of EPO, HIF-1α, HIF-2α, and VEGF was examined in kidney and liver tissues of Tibetan and plain sheep. The results showed that the protein and mRNA of the four factors were expressed in each tissue, and the expression of each factor differed in each tissue.

By examining the expression of EPO protein, we found that different regions and cells of Tibetan and plain sheep expressed different levels of EPO protein, which was present in the renal tubular epithelial cells of both Tibetan and plain sheep. For the glomerular cytoplasm, Tibetan sheep showed positive and higher expression, whereas plain sheep did not, suggesting that different cells have different degrees of hypoxia perception. EPO is not the only expression factor that promotes the regulation of hypoxia, and many hormones in the organism, such as EPOR or hematopoietic cell phosphatase inhibitors, can stimulate the production of EPO, resulting in the selective expression of EPO in cells that perform their respective regional functions (Chong et al., 2002). Although the kidneys are the main source of EPO in the body, the liver is also a major producer of EPO. We found that EPO showed positive signals in the interlobular veins, interlobular arteries, and interlobular bile ducts in Tibetan sheep, while plain sheep showed positive signals only in the interlobular veins and interlobular arteries, with some differences between the two, suggesting that EPO can adapt to external hypoxic conditions and that hypoxia promotes hepatic expression. Prolonged hypothermia increases oxygen consumption in the kidneys and liver, thereby increasing the oxygen concentration in the organs, which in turn causes the intrinsic cells of the kidneys and liver to present a hypoxic state. It was found that the expression of EPO in the kidneys and liver increased continuously with time, mainly because hypoxia-induced the expression of HIF-1α, which in turn controlled the secretion of EPO, and finally caused a continuous increase in EPO in tissues, which also suggests that the pathway of " hypoxia→HIF-1α→EPO→RBC→hypoxia increase " was related (Debevec et al., 2012).

HIF-1α and HIF-2α can activate the expression of HIF- induced EPO, which further increases the oxygen-

Table 2. Average IOD values of EPO, HIF-1α, HIF-2α, and VEGF in kidney and liver tissues of Tibetan and plain sheep (Mean ± SD).

	EPO	HIF-1α	VEGF	HIF-2α
Tibetan sheep kidney	0.4867±0.0293	0.4413±0.024	0.3914±0.0120	0.2475±0.0076
Plain sheep kidney	0.3035±0.0078	0.3285±0.0068	0.2927±0.0126	0.2039±0.0087
Tibetan sheep liver	0.6374±0.0166	0.4366±0.0232	0.3317±0.0272	0.2312±0.0058
Plain sheep liver	0.4167±0.1830	0.3494±0.008	0.2796±0.0137	0.1403±0.0068

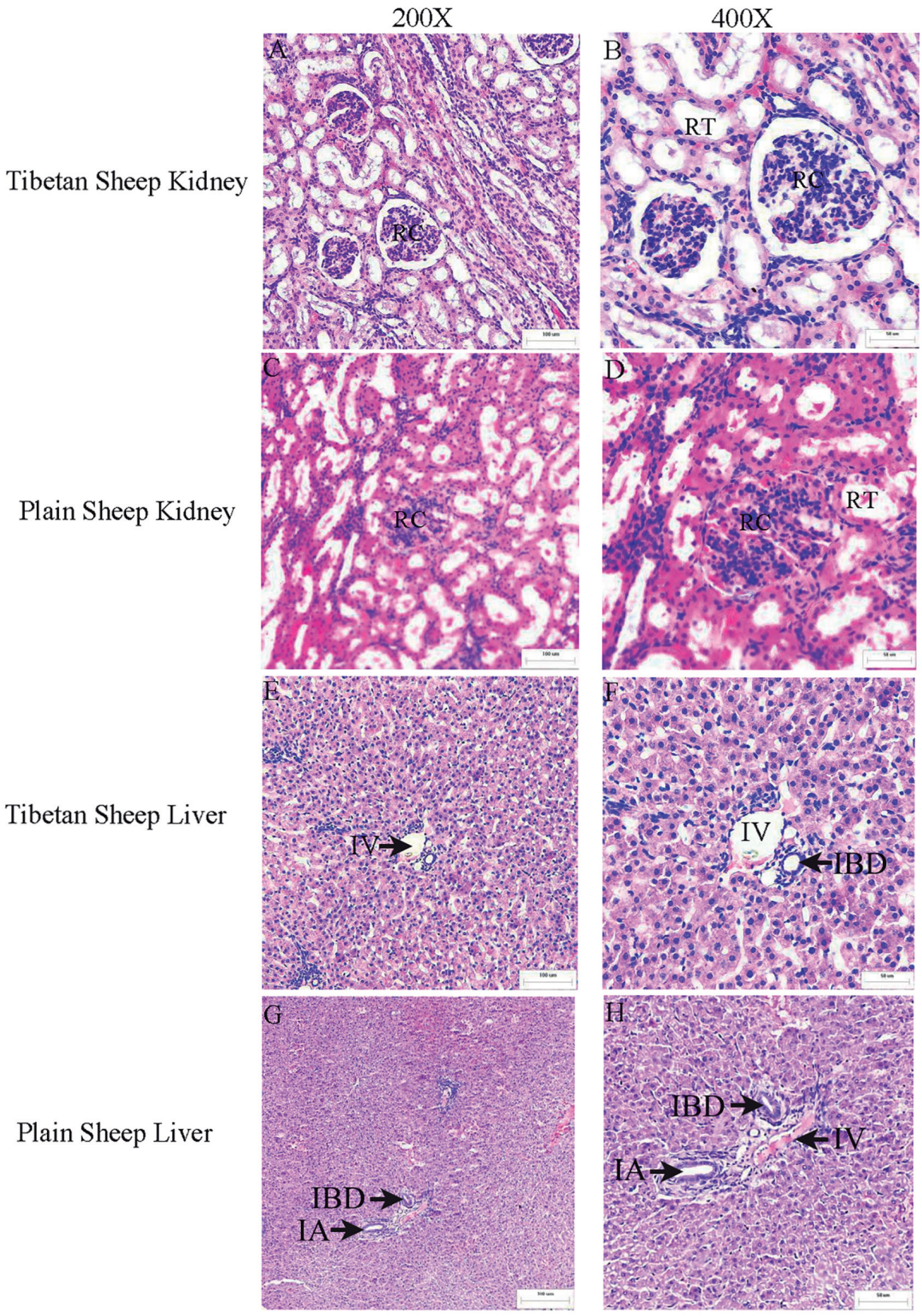

Fig. 1. Results of Hematoxylin and eosin staining (H&E) staining of Tibetan and plain sheep. **A, B.** HE staining of Tibetan sheep kidney tissues at different magnifications of the microscope. **C, D.** HE staining of plain sheep kidney tissues at different magnifications of the microscope. **E, F.** HE staining of Tibetan sheep liver tissues at different magnifications of the microscops. **G, H.** HE staining of plain sheep liver tissues at different magnification of the microscops. RT: Renal tubule; RC: Renal corpuscle; IBD: Interlobular bile duct; IV: Interlobular vein; IA: Interlobular artery. A, C, E, G, x 200; B, D, F, H, x 400.

delivery capacity of erythrocytes and promotes vascular regeneration and reconstruction (Wenger et al., 2005). HIF-1α, HIF-2α, and VEGF were all expressed in kidney cells, but their expression was stronger in Tibetan sheep than in plain sheep, suggesting that hypoxia at low temperature promotes protein expression in the kidney region. In addition, HIF-1α, HIF-2α, and VEGF were expressed in the interlobular bile duct epithelial cells hepatocytes mainly in the cytoplasm around the hepatic sinusoidal veins, and partially in the nucleus of Tibetan and plain sheep. This could be because of the presence of oxygen-sensing regulation during pure hypoxia, the

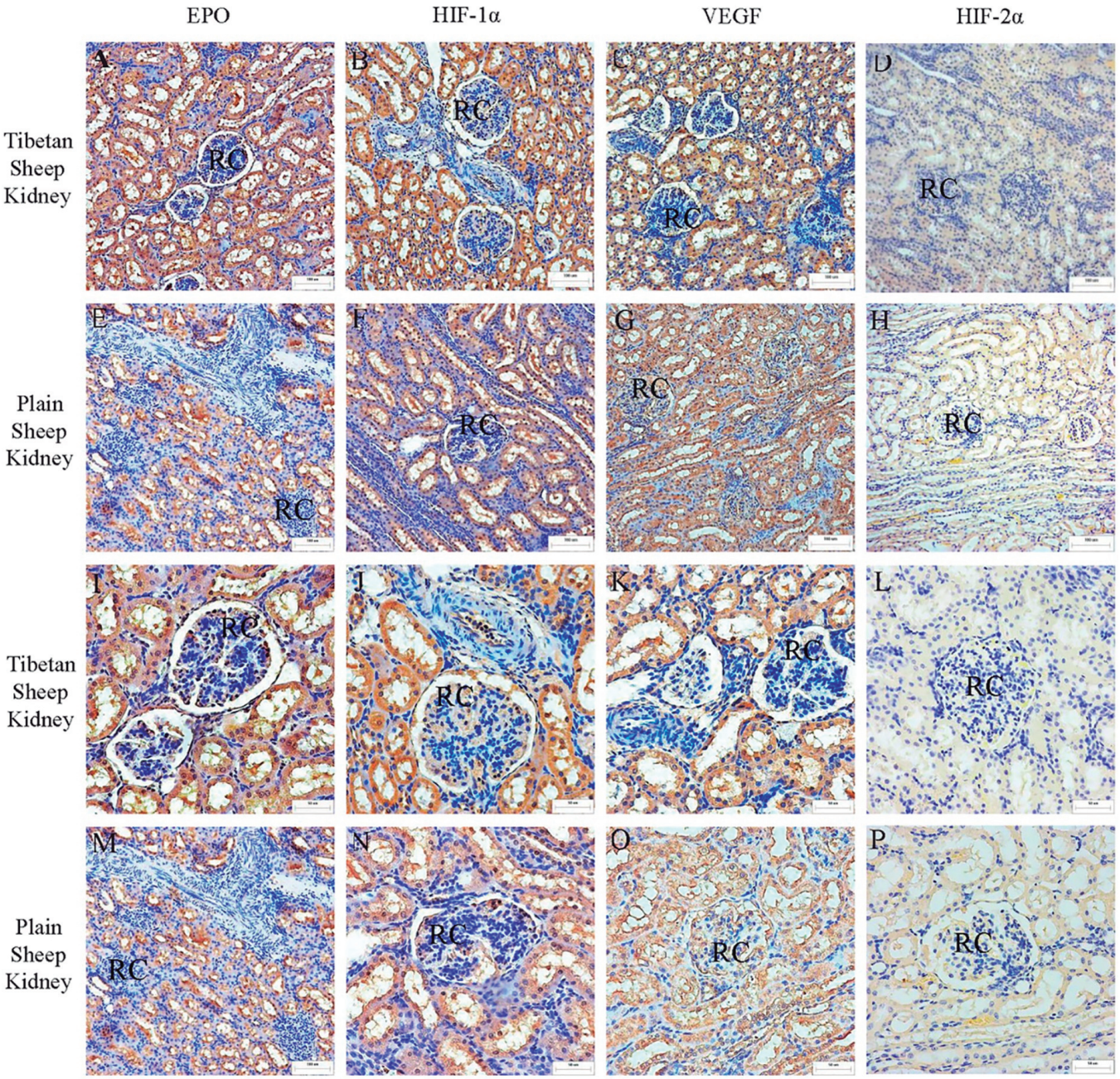

Fig. 2. Immunohistochemical staining of EPO, HIF-1α, HIF-2α, and VEGF in the kidney of Tibetan and plain sheep. **A-D, I-L.** the distribution and expression of EPO, HIF-1α, HIF-2α, and VEGF in Tibetan sheep kidney tissues at different magnifications of the microscope. **E-H, M-P.** The distribution and expression of EPO, HIF-1α, HIF-2α, and VEGF in pPlain sheep kidney tissues at different magnifications of the microscope. RT: Renal tubule; RC: Renal corpuscle. A-D, E-H, x 200 I-L, M-P, x 400.

presence of an oxygen- pressure-dependent degradation domain within the hypoxic regulator that can be rapidly degraded under normoxic conditions, and the fact that hypoxia increases the stability of the protein, resulting in higher levels. In conclusion, HIF-1α, HIF-2α, and VEGF were co-expressed with EPO (Maeno et al., 2005) in both the kidneys and liver, suggesting that their production is likely to be regulated by HIF-1α, HIF-2α, or VEGF.

We also examined the mRNA expression levels of EPO, HIF-1α, HIF-2α, and VEGF. The expression trend of HIF-1α mRNA in Tibetan sheep converged with that

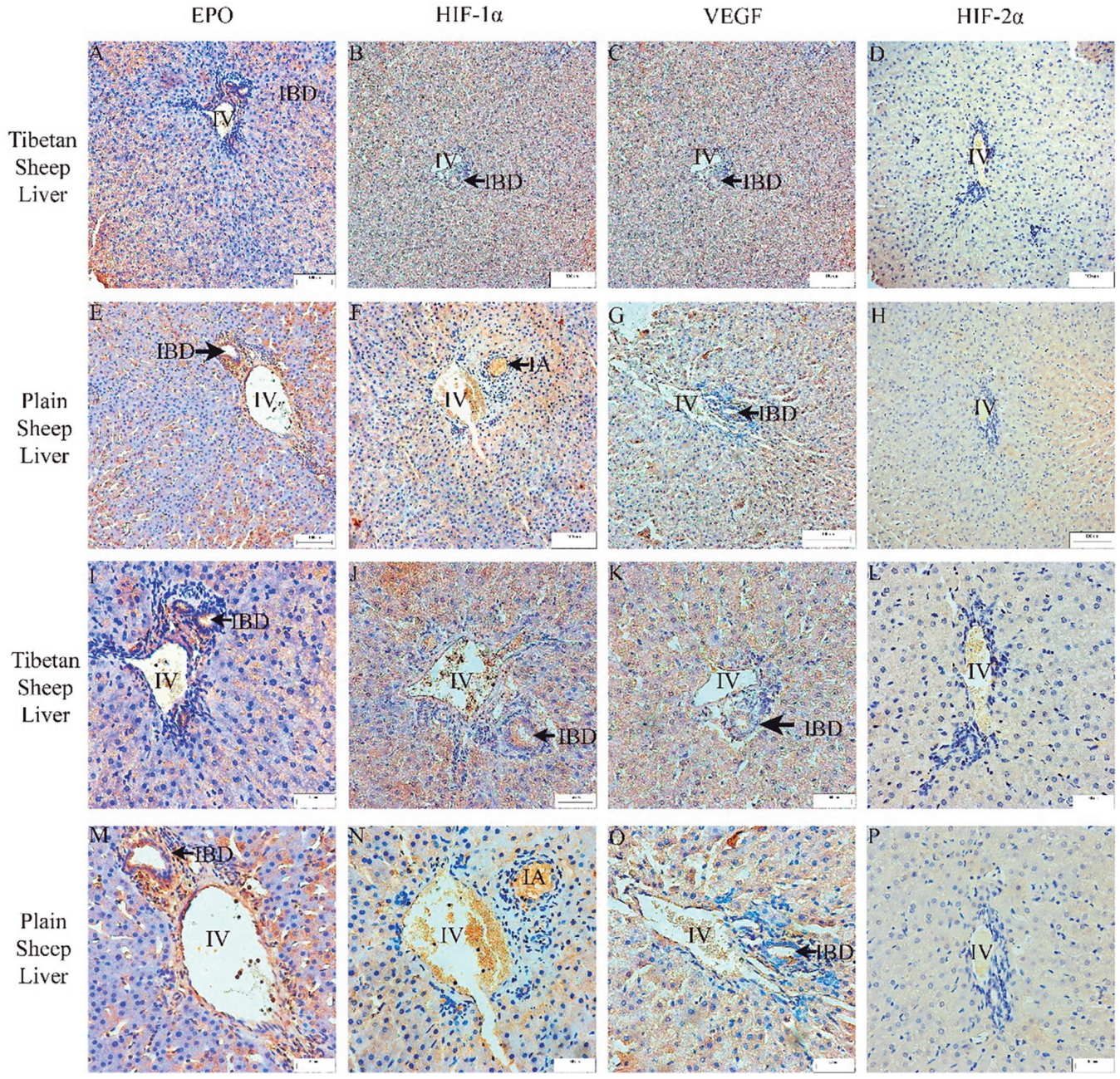

Fig. 3. Immunohistochemical staining of EPO, HIF-1α, HIF-2α, and VEGF in the liver of Tibetan and plain sheep. **A-D, I-L.** The distribution and expression of EPO, HIF-1α, HIF-2α, and VEGF in Tibetan sheep liver tissues at different magnifications of the microscope. **E-H, M-P.** The distribution and expression of EPO, HIF-1α, HIF-2α, and VEGF in plain sheep liver tissues at different magnifications of the microscope. IBD: Interlobular bile duct; IV: Interlobular vein; IA: Interlobular artery. A-D, I-L, x 200 E-H, M-P, x 400.

of HIF-2α, whereas that of HIF-2α and VEGF in plain sheep converged. Notably, we could not detect EPO mRNA expression in the kidneys and liver in either Tibetan or plain sheep, which is consistent with the results of Darby; the main reason for this may be due to the relatively widespread low-level expression of EPO in the liver and the low number of cells expressing EPO in the kidney (Darby et al., 1995). Detection of the human EPO transgene in the livers of transgenic mice after hemorrhage has been reported, but high expression of the endogenous EPO gene in these animals is difficult to detect (Koury et al., 1991). In this experiment, the mRNA expression trends of EPO, HIF-1α, HIF-2α, and VEGF in tissues were not consistent with the protein expression trends. The immunohistochemistry results showed the highest expression of EPO, and the RT-qPCR results showed the highest expression of HIF-1α. We believe that the expression of different factors in cells after transcription and translation into proteins depends mainly on the rate of degradation and does not necessarily coincide exactly with their mRNA expression (Xiong et al., 2015). Therefore, the differences in mRNA and protein expression in the results of this study may be related to this.

Hypoxia affects kidney cells with altered function. Acute hypoxia can lead to renal tubular damage, causing

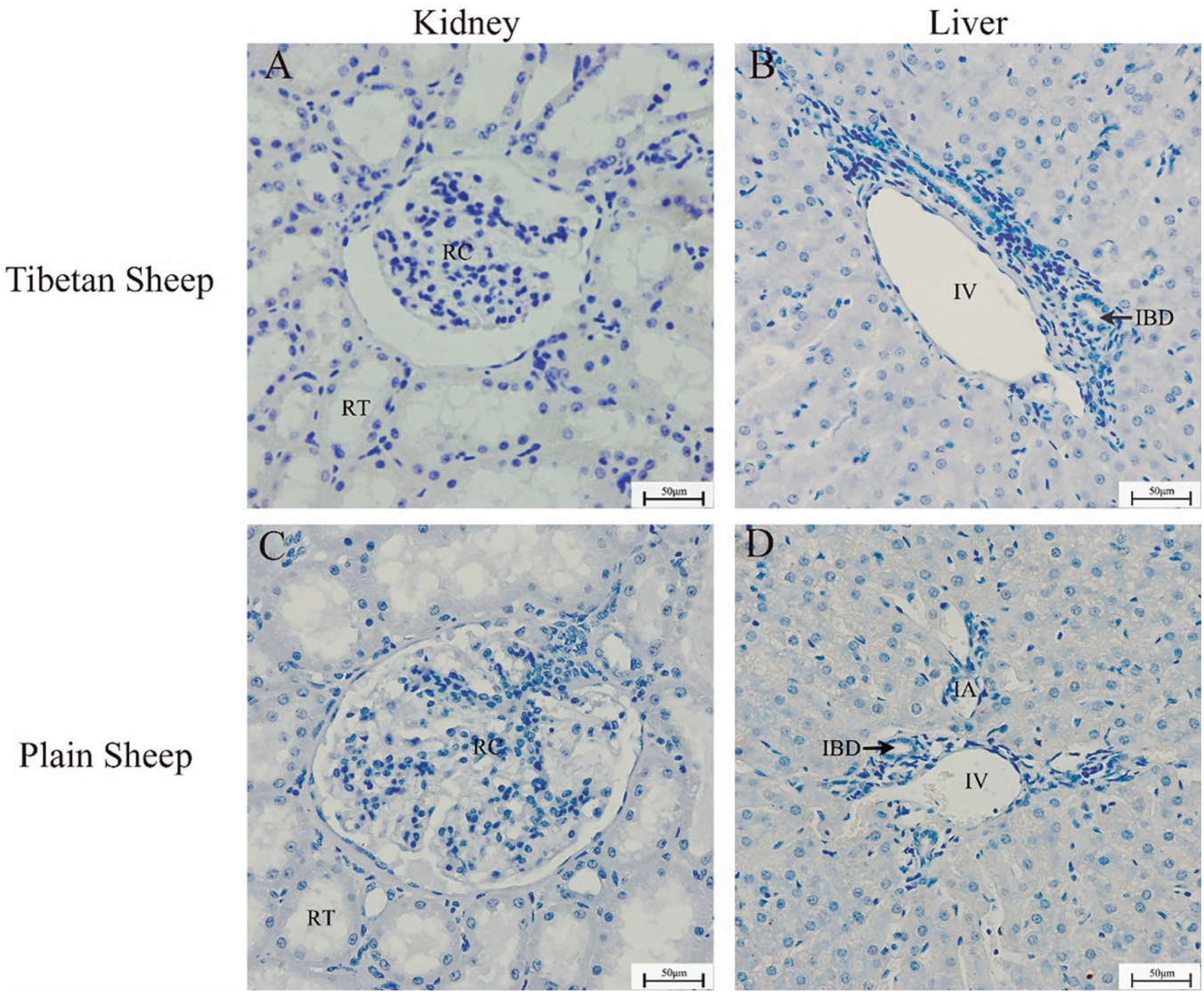

Fig. 4. Blank control of Tibetan and plain sheep tissues. **A, B.** Blank controls of kidney and liver tissues of Tibetan sheep, respectively. **C, D.** Blank controls of kidney and liver tissues of plain sheep, respectively. RT: Renal tubule; RC: Renal corpuscle; IBD: Interlobular bile duct; IV: Interlobular vein; IA: Interlobular artery. x 400.

changes in kidney function and apoptosis (Malhotra et al., 2008). Long-term hypoxia produces a chronic renal inflammatory reaction, leading to glomerulosclerosis, tubulointerstitial fibrosis, and reduced density of glomeruli and peritubular vascular network, further aggravating renal tissue hypoxia and kidney damage, forming a vicious cycle, eventually leading to renal insufficiency (Vasanth Rao et al., 2019). Chronic hypoxia can lead to intrahepatic vascular congestion, distension of blood vessels, poor intrahepatic microcirculation, impaired hepatic tissue nutrition, and altered hepatocyte membrane permeability, resulting in hepatocyte congestion, edema, and steatosis (Maiti et al., 2008). Acute low-pressure hypoxia and its associated oxidative stress are also important aspects of the pathogenesis of acute plateau disease (Dosek et al.,

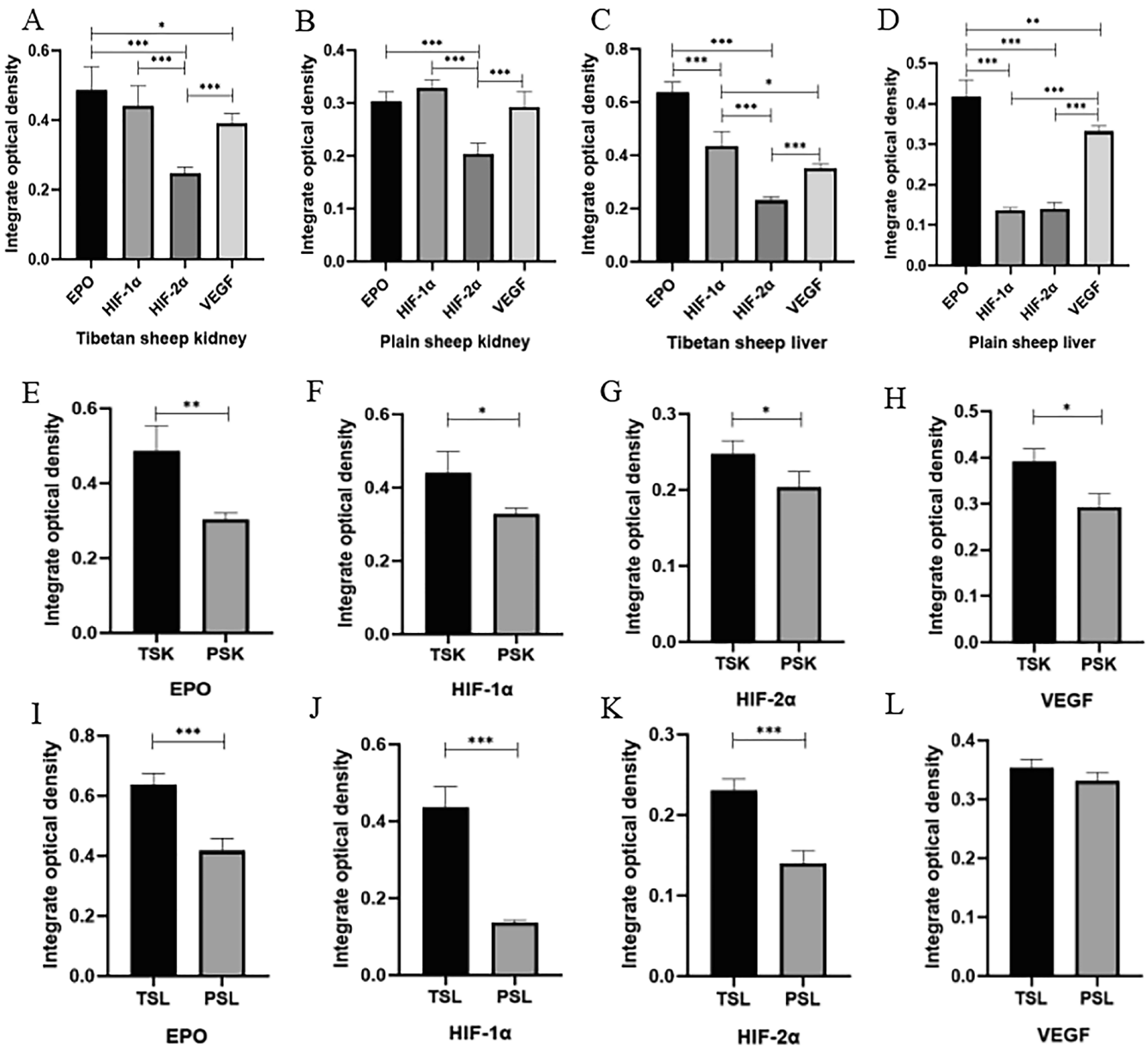

Fig. 5. Integrated optical density (IOD) value analysis. **A.** The IOD analysis of Tibetan sheep kidney tissues, plain sheep kidney tissues **(B)**, Tibetan sheep liver tissues **(C)**, and plain sheep liver tissues **(D)**. **E-H.** The comparison of IOD values of EPO, HIF-1α, HIF-2α, and VEGF in Tibetan and plain sheep kidney tissues. **I-L.** The comparison of IOD values of EPO, HIF-1α, HIF-2α, and VEGF in Tibetan and plain sheep liver tissues. **P*<0.05, ***P*<0.01, ****P*<0.001

2007).

In this study, we explored the expression of EPO, HIF-1α, HIF-2α, and VEGF in both the kidney and liver of Tibetan and plain sheep, which is consistent with the results of human and murine studies where EPO was correlated with HIF-1α, HIF-2α, and VEGF in both the liver and kidney of Tibetan and plain sheep ($P<0.05$). This suggests that the plateau animal, Tibetan sheep, live in a low-oxygen region where HIF-1α, HIF-2α, VEGF, and EPO have established a stable regulatory relationship. It is similar to that of low-altitude sheep, which is the most successful biological marker of genetic adaptation in highland animals. Clarifying the expression of the factors in the liver and kidney of Tibetan sheep is important to reveal the hypoxic adaptation of Tibetan sheep and can also lay the foundation for the study of the related factors in various tissues and their host protective effects, whose specific regulatory mechanisms and generating cell types are our next directions for research.

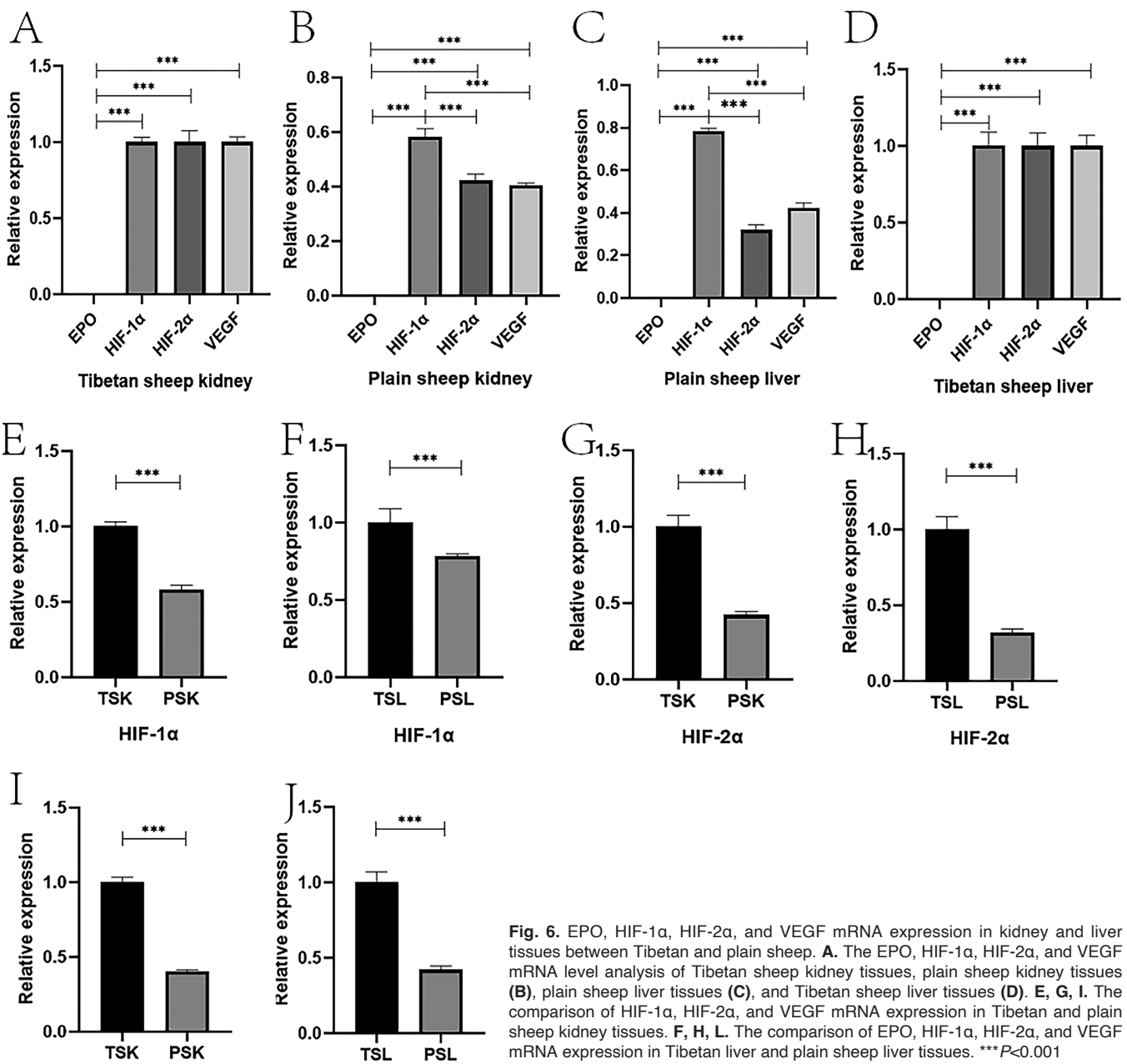

Fig. 6. EPO, HIF-1α, HIF-2α, and VEGF mRNA expression in kidney and liver tissues between Tibetan and plain sheep. **A.** The EPO, HIF-1α, HIF-2α, and VEGF mRNA level analysis of Tibetan sheep kidney tissues, plain sheep kidney tissues **(B)**, plain sheep liver tissues **(C)**, and Tibetan sheep liver tissues **(D)**. **E, G, I.** The comparison of HIF-1α, HIF-2α, and VEGF mRNA expression in Tibetan and plain sheep kidney tissues. **F, H, L.** The comparison of EPO, HIF-1α, HIF-2α, and VEGF mRNA expression in Tibetan liver and plain sheep liver tissues. ***$P<0.001$

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Declaration of Competing Interest. The authors declare no conflict of interest.

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