

Insulin promotes sinusoidal endothelial cell proliferation mediated by upregulation of vascular endothelial growth factor in regenerating rat liver after partial hepatectomy

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Abstract

AIM: To determine whether insulin could promote sinusoidal endothelial cell (SEC) proliferation mediated by upregulation of vascular endothelial growth factor (VEGF) in regenerating rat liver after partial hepatectomy (PHx).

METHODS: Adult male Sprague-Dawley rats undergoing 70% PHx were injected with insulin (300 MU/kg) or saline via the tail veins every 8 h after surgery for 7 d and killed at 0, 24, 48, 72, 96, 120, 144, and 168 h after surgery. Proliferation of both hepatocytes and SECs was monitored by evaluating the proliferating cell nuclear antigen (PCNA) labeling index (LI). The expression of VEGF protein was evaluated by immunohistochemistry. The mRNA expressions of VEGF and its receptors Flt-1 and Flk-1 were evaluated by semi-quantitative reverse transcription-PCR.

RESULTS: Insulin markedly increased the expression of VEGF mRNA between 24 and 120 h after hepatectomy compared to controls. Similarly, insulin significantly increased the expression of Flt-1 between 24 and 96 h. However, insulin had no significant effect on Flk-1. Furthermore, the immunohistochemical staining revealed that expression of VEGF protein increased in the insulin groups. Insulin significantly increased the PCNA LI of hepatocytes and SECs compared to controls.

CONCLUSION: Exogenous insulin may promote SEC proliferation with an enhanced expression of VEGF and its receptor Flt-1 in regenerating rat liver after PHx.

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Key words: Insulin; Sinusoidal endothelial cell; VEGF

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INTRODUCTION

Hepatic regeneration is a key step in the recovery process that occurs after various forms of liver injury, including partial hepatectomy (PHx)^[1]. The exact mechanism of liver regeneration remains unclear. Several important cytokines and growth factors are involved in the regulation of hepatocyte proliferation^[2-6]. Angiogenesis is a fundamental course required for wound healing and regeneration^[5]. The liver microvasculature consists of large vessels such as portal and central venules and hepatic arterioles lined with continuous endothelial cells, and sinusoids lined with sinusoidal endothelial cells (SECs) expressing undiaphragmed fenestrations^[6]. SECs are the second largest number of resident liver cells, and are vital in supplying nutrients and growth factors to proliferating hepatocytes by the formation of new blood vessels during liver regeneration^[5]. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor stimulating the proliferation and migration of endothelial cells, and is also known as a vascular permeability factor^[7-9]. The effects of VEGF are mediated through at least two specific receptors, Flt-1 and Flk-1, expressed on the endothelial cell surface^[7]. Recent studies have revealed that VEGF stimulates endothelial cell proliferation and plays an important role in the supply of blood to the newly replicating hepatocytes during regeneration^[5,7]. Moreover, it is reported that exogenous VEGF can stimulate liver cell proliferation following PHx^[5,7,10].

Insulin is a hepatocyte growth modulator, which is essential in liver regeneration, and is transported into the liver during the process of liver regeneration^[11]. There is evidence that treatment with insulin contributes to the normal regeneration of liver by decreasing hepatocellular injury and by increasing hepatocyte proliferative capacity^[11-13]. In addition, insulin therapy has also been shown epidemiologically as an independent risk factor for the progression of intraocular neovascularization in diabetic retinopathy^[13,14]. Yamagishi *et al.*^[13] have demonstrated that insulin stimulates the growth and tube formation of human microvascular endothelial cells through autocrine VEGF *in vitro*. Nevertheless the effect of insulin on blood vessels during regeneration after PHx in rats is still unknown.

Based on these studies, we believe that insulin promotes SECs and subsequently liver regeneration by accelerating the expression of VEGF. In the present study, we investigated the expression of VEGF and its receptors, Flt-1 and Flk-1, as well as the expression of proliferating cell nuclear antigen (PCNA) during rat liver regeneration after 70% PHx.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats aged 7–8 wk (180–200 g; from the Center of Animal Laboratory, Wuhan University) were used for all experiments. The rats were maintained in temperature-controlled rooms in a 12-h light/dark cycle with free access to food (standard laboratory chow) and water. Seventy percent PHx was performed by the standard two-thirds PHx under light anesthesia with ether. The animals were randomly divided into two groups: PHx group was injected with insulin (300 MU/kg) in saline via the tail vein of rats every 8 h after PHx for a week, while control group was given saline alone. Five rats in each group were used at each of the eight time points (0, 24, 48, 72, 96, 120, 144, and 168 h after surgery, with a total of 80 animals). All surgeries were performed between 08:30 and 11:30 a.m..

Immunohistochemical examination of VEGF

Anti-VEGF antibody (Santa Cruz Biotechnology Inc., Delaware, CA, USA) was used for the immunohistochemical staining of VEGF on formalin-fixed and paraffin-embedded liver tissue. Briefly, liver tissues excised at designated times after surgery were fixed in 40 g/L formaldehyde solution and embedded in paraffin wax. Sections were cut at 4 μ m, mounted on poly-L-lysine-coated glass slides, air dried overnight at room temperature, dehydrated in graded alcohols, and cleared in xylene. The three-step immunoperoxidase method using a LSAB kit (Dako, Copenhagen, Denmark) was performed according to the manufacturer's instructions. Anti-VEGF antibody used was diluted at 1:100 and incubated overnight at 4 $^{\circ}$ C.

PCNA labeling index

Immunohistochemical staining of the PCNA was performed using an indirect two-step labeling technique with peroxidase-conjugated IgG (Amersham NA 931, Amersham Life Science, NY, USA). The monoclonal anti-PCNA antibodies were purchased from Amersham Life Science (PC-10). The sections were deparaffinized in xylene and dehydrated by passage through graded alcohol. Endogenous peroxidase was blocked by incubation of the slides with 3% H₂O₂ in methanol for 30 min. After being washed in PBS, 10% normal goat serum (Amersham Life Science) in PBS was applied for 20 min at room temperature. Slides were incubated overnight with monoclonal anti-PCNA antibodies and subsequently exposed to prediluted peroxidase-conjugated IgG for 40 min at room temperature. The peroxidase reaction was developed by incubation in 0.005% H₂O₂ and 0.02% 3,3'-diaminobenzidine tetrachloride. The proliferative activity in the remnant liver was expressed as the labeling index (LI). The LI was the ratio of the number of PCNA positive nuclei of either hepatocytes or SECs to the total

number of cells counted. In each liver, the hepatocytes and SECs in 200 consecutive high power fields were counted. In this study, the spindle-shaped sinusoid-lining cells in the open sinusoids were regarded as SECs.

RNA extraction, cDNA synthesis and PCR amplification of different angiogenic factors

About 20 mg liver tissues was immediately flash frozen in liquid nitrogen and stored at -70° C after excision for RNA isolation. Total RNA was extracted with the RNeasy[®] Mini kit (Qiagen Cat. No. 74104) according to the manufacturer's protocol and eluted in 25 μ L RNase-free water. Single-stranded cDNA was synthesized from 8 μ L of RNA by the RevertAid[™] first strand cDNA synthesis kit (MBI Cat. No. K1621) with random hexamer primers and 3 μ L of the product was used for PCR.

The PCR primer pairs are listed in Table 1. Thirty cycles of DNA amplification using a Taq DNA polymerase (recombinant, MBI Cat. No. EP0404) was performed under the following conditions: denaturation at 94 $^{\circ}$ C for 1 min, annealing (β -actin, 52 $^{\circ}$ C; VEGF, 60 $^{\circ}$ C; Flt-1, 53 $^{\circ}$ C; Flk-1, 52 $^{\circ}$ C) for 1 min, extension at 72 $^{\circ}$ C for 1 min. Negative controls (cDNA-free solutions) were included in each reaction. β -actin, a house-keeping gene equally expressed in most eukaryotic cells, was used as an external standard and amplified in parallel with different angiogenic factors in all PCR samples.

Table 1 Oligonucleotides used as primers in RT-PCR procedure

Primer	Nucleotide sequence	PCR product lengths (bp)
Rat VEGF	Sense: 5'-ACTGGACCTCGGCTTACTG-3'	256
Antisense:	5'-ACGCACTCCAGGGCTTCATC-3'	
	GenBank NM031836	
Rat Flt-1	Sense: 5'-AGGAGAGGACCTGAACTGTCTT-3'	214
Antisense:	5'-ATTCTGGGCTCTGAGGCATAG-3' ^[10]	
Rat Flk-1	Sense: 5'-GTGATTGCCATGTCTTCTGGC-3'	337
Antisense:	5'-TCAGACATGAGAGCTCGATGCT-3'	
	GenBank U93306	
Rat β -actin	Sense: 5'-TTCCACACACACCAGCTTCG-3'	366
Antisense:	5'-GGGGTGGTGTGGAGATTAG-3'	
	GenBank NM031144	

Semi-quantification of PCR products

After PCR amplification from 50 μ L reaction volume, 10 μ L was electrophoresed on 1.5% agarose gel in 1 \times TBE buffer. The amplified bands were detected by ethidium bromide staining. The intensity of ethidium bromide fluorescence was measured by the Photo Documentation and Imaging System (Bio-1D, VL, France). For quantification of the PCR products from all samples, the samples were evaluated by comparing the PCR product to β -actin.

Statistical analysis

The results were expressed as mean \pm SD. Statistical analysis was performed for the unpaired data using SPSS11.0 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Hepatic VEGF, Flt-1 and Flk-1 mRNA expression

The changes in hepatic VEGF, Flt-1 and Flk-1 mRNA following surgery are shown in Figures 1 and 2. The expression of VEGF mRNA increased after 24 h of 70% PHx, with a peak from 48 to 72 h. Insulin significantly increased VEGF mRNA from 24 to 120 h after hepatectomy compared to controls ($P<0.01$; Figures 1C, D and 2A). Similarly, hepatic Flt-1 mRNA expression increased after hepatectomy. Insulin significantly increased Flt-1 mRNA from 24 to 96 h after hepatectomy compared to controls ($P<0.01$; Figures 1E, F and 2B). Insulin had no significant

effect on Flk-1 when compared to controls (Figures 1G, H and 2C).

PCNA labeling index

The PCNA LI was lower than 5% in both hepatocytes and SECs before hepatectomy. However, liver cell proliferation was evident as early as 24 h postoperation. Insulin significantly promoted hepatocyte proliferation from 24 to 120 h compared to controls ($P<0.01$, Figure 3).

Immunohistochemical staining of VEGF protein in remnant liver

Before hepatectomy, there were very few VEGF-positive

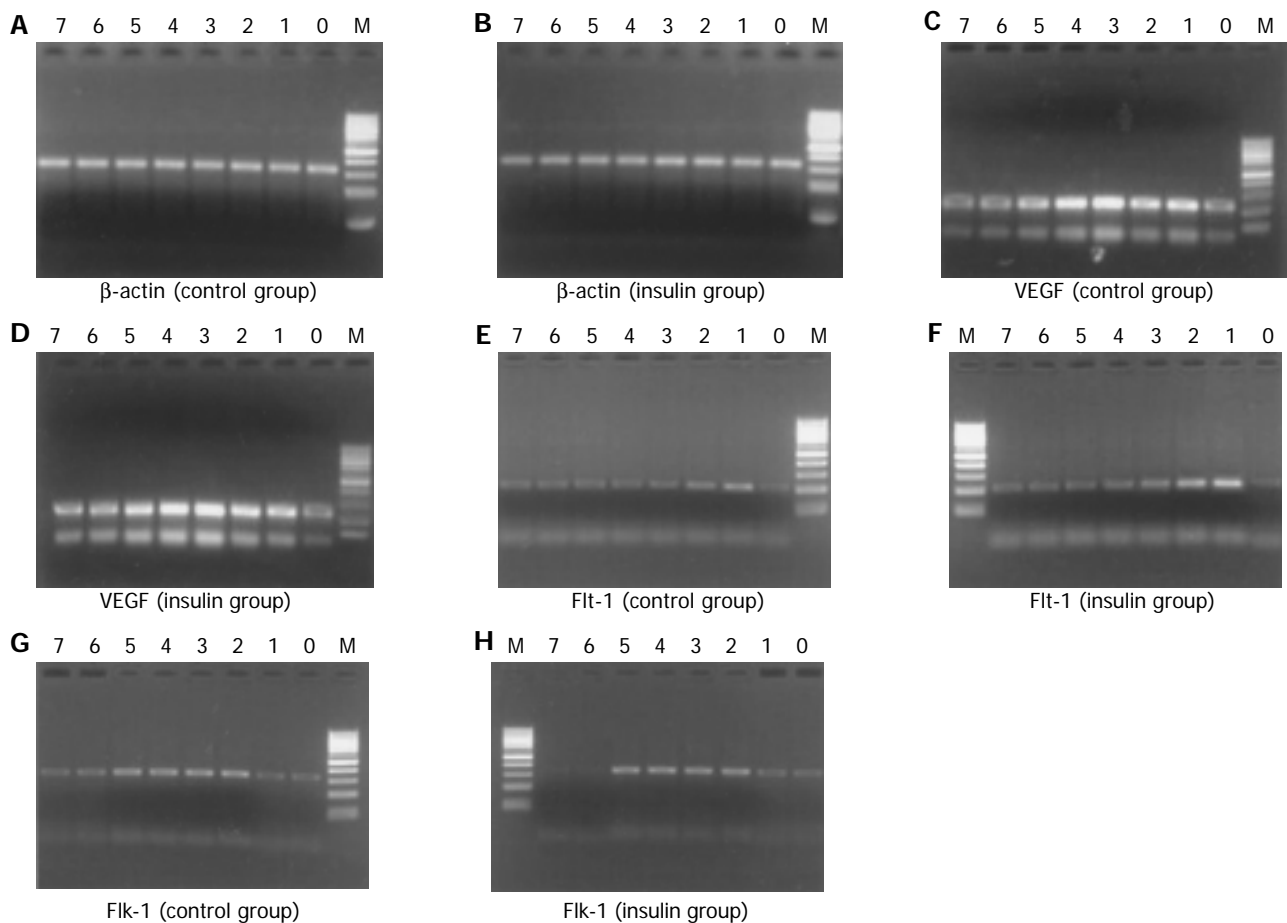


Figure 1 RT-PCR results of VEGF, Flt-1, and Flk-1 mRNA expressions in liver of different groups. M: 100-bp DNA ladder (upper to lower: 1 000, 900, 800,

700, 600, 500, 400, 300, 200, and 100 bp); lane 0: 0 h; lane 1: 24 h; lane 2: 48 h; lane 3: 72 h; lane 4: 96 h; lane 5: 120 h; lane 6: 144 h; lane 7: 168 h.

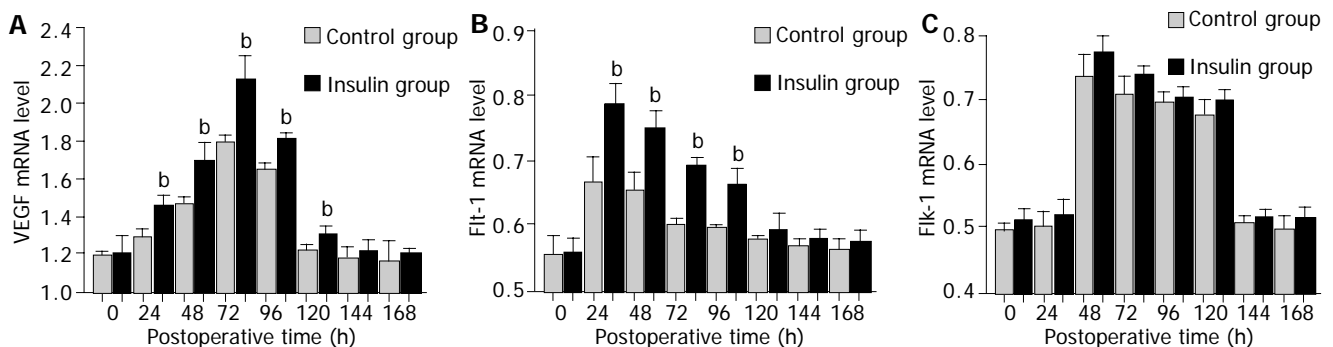


Figure 2 VEGF (A), Flt-1 (B), and Flk-1 (C) mRNA expressions after 70%

hepatectomy in rats by RT-PCR.

cells, but increased expression of VEGF was observed 24 h after surgery ($P<0.01$, Figure 4A). Insulin increased the expression of VEGF, with a peak at 72 h ($P<0.01$, Figure 4D).

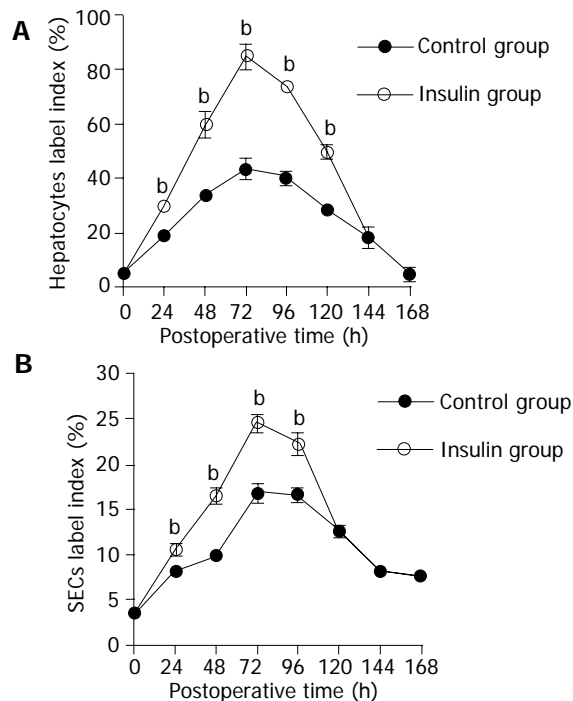


Figure 3 PCNA LI of hepatocytes (A) and SECs (B) after 70% hepatectomy in rats.

DISCUSSION

Liver remodeling following PHx comprises a complex set

of events including activation and secretion of numerous factors, cell proliferation, thus finally re-establishing liver architecture^[15-19]. Angiogenesis is the integrated process of endothelial cell division, vascular sprouting, and migration from pre-existing cells^[20], indicating that SEC proliferation is strongly associated with hepatocyte proliferation during liver regeneration. Liver regeneration involves two processes: liver architecture remodeling and liver cell proliferation. In our study, SECs influenced the regenerative capacity of liver following PHx. Rebuilding the network of blood supply is necessary in liver regeneration. SECs play an important role in the formation of a complex network of blood vessels for remodeling liver architecture following liver resection. Additionally, SECs surround avascular clusters of hepatocytes, subsequently re-establish normal vascular architecture and directly supply nutrition and growth factors to liver cells^[6].

The role of VEGF in liver regeneration has been reported^[20-25]. SECs have a unique response to growth factors^[6-8,18]. Information on the association of angiogenic factors with liver regeneration is available^[5,6,18]. VEGF isoform signal has several receptors, including Flt-1 and Flk-1^[26-30]. These receptors can operate in a paracrine and cooperative manner with VEGF produced by hepatocytes in regulating SEC growth and subsequent vascularization of the liver^[13]. The receptors are structurally similar to members of the platelet-derived growth factor receptor family and consist of an extracellular domain composed of seven immunoglobulin-like motifs, a transmembrane domain, a juxtamembrane domain, a tyrosine kinase that is split by a kinase insert region, and a C-terminal tail. It is uncertain what role each VEGF receptor actually plays in cell signaling during liver revascularization. In some studies, Flk-1/KDR and Flt-1 have been shown to act in unique capacities^[6]. These studies have determined that Flk-1/KDR can activate both mitogenicity and motility pathways, whereas

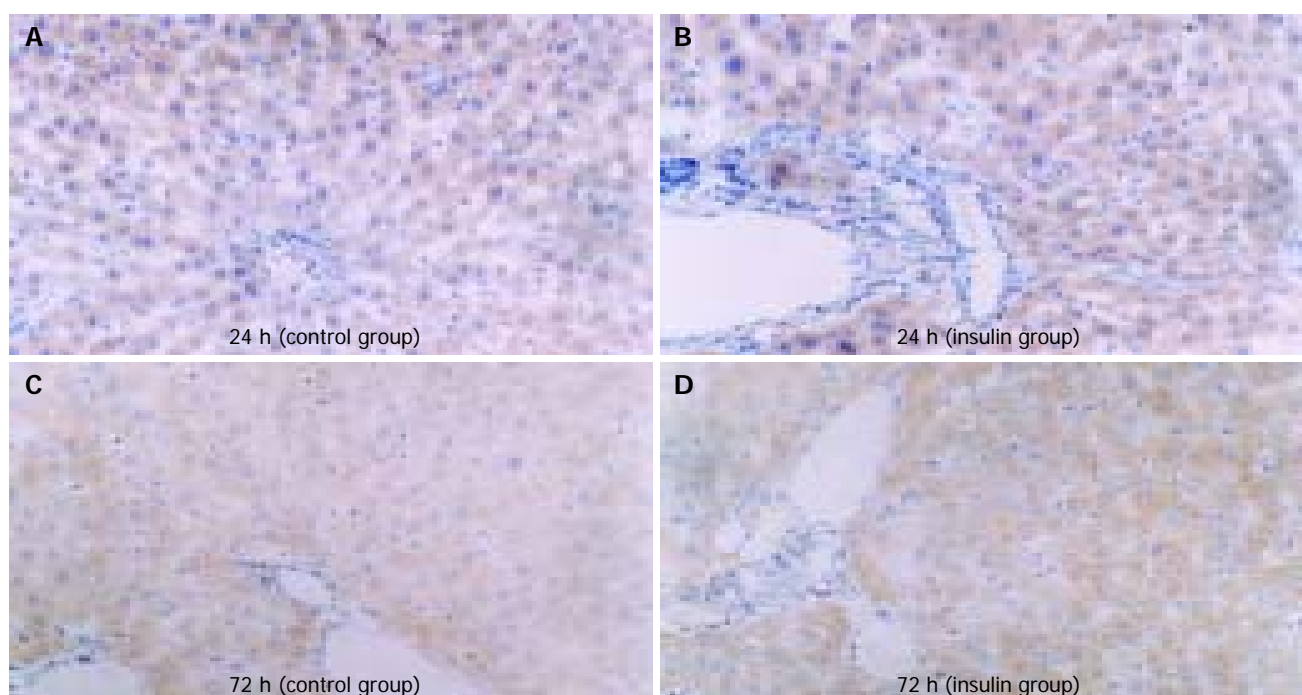


Figure 4 Expression of VEGF protein at 24 h in control group (A) and insulin

group (B), at 72 h in control group (C), and insulin group (D).

Flt-1 appears to mediate actin reorganization^[6]. Moreover, Flt-1 is induced by hypoxia, which emerges during regeneration, whereas Flk-1/KDR is not^[6]. Exogenous VEGF₁₆₅ can stimulate liver cell proliferation following PHx and antibodies to VEGF significantly inhibit hepatocyte proliferation following PHx^[5,29]. However, VEGF₁₆₅ does not induce either a motogenic or a mitogenic response in isolated hepatocytes, though Flt-1 is phosphorylated^[6,29], indicating that VEGF may stimulate liver cell proliferation by acting on surrounding SECs, which influence hepatocytes by improving nutrient availability or through vascular permeability.

The present study demonstrated that exogenous insulin could stimulate SECs following PHx in rats. Insulin can induce a host of effects on glucose transport and utilization, protein synthesis, and cell proliferation, *etc.*^[12].

However, the effect of insulin on blood vessels during regeneration after PHx in rats is still unknown. It has been reported that vascular abnormalities and pathologies are commonly associated with diabetes^[31,32]. Jiang *et al.*^[25], demonstrated that insulin can stimulate several signaling cascades in endothelial cells and vascular smooth muscle cells by phosphorylating both insulin receptor substrate-1 and -2. The vascular-specific action of insulin may be attributed to the growth factors, especially VEGF, and cytokines of which the expression is regulated by insulin. The exact mechanism underlying insulin-induced VEGF mRNA upregulation in microvascular EC is currently unknown. It was reported that insulin-induced VEGF expression is mediated through the activation of phosphatidylinositol (PI) 3-kinase/Akt being downstream to the insulin receptors^[33,34]. It was reported that the activation of PI 3-kinase/Akt pathway can upregulate the expression of hypoxia-inducible factor-1 α , a principal factor that causes angiogenesis^[34]. Insulin may upregulate the VEGF mRNA through a nuclear factor- κ B-mediated pathway, and mAb against VEGF can completely neutralize the proliferation and tube formation of endothelial cells induced by insulin^[11,33].

In conclusion, insulin induces expression of VEGF and its receptor after PHx, and promotes liver regeneration besides the traditional metabolic action on hepatocytes.

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