Research Paper

p38 Mitogen-activated protein kinase mediates hypoxia-induced vascular endothelial growth factor release in human endothelial cells

FAN Bei, WANG Yan-Xia, YAO Tai, ZHU Yi-Chun*
Department of Physiology and Pathophysiology, Key Laboratory of Molecular Medicine of the Ministry of Education, Fudan University Shanghai Medical College, Shanghai 200032, China.

Abstract: Increased vascular endothelial growth factor (VEGF) biosynthesis in vascular endothelial cells has been reported to play an obligatory role in promoting angiogenesis. Nevertheless, the intracellular signaling mechanisms of hypoxia-induced VEGF release remain largely unknown. Human umbilical vein endothelial cell lines (ECV304) were cultured in normoxic or hypoxic conditions for 12~24 h and harvested for determination of VEGF mRNA expression and phosphorylation of ERK1/2 and p38 mitogen-activated protein kinase (p38 MAPK) by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis, respectively. Secreted VEGF protein was measured by enzyme-linked immunosorbent assay (ELISA). It has reported that PD98059, an ERK inhibitor, was able to blunt the hypoxia-induced activation of the expression of VEGF gene. In accordance with this report, an increase in ERK1/2 phosphorylation and VEGF biosynthesis was observed in ECV304 cells cultured in hypoxia, and this increase was blocked by PD98059. The novel finding of the present study is that an activation of p38 MAPK is involved in hypoxia-induced increase in VEGF biosynthesis. SB202190, an inhibitor of p38 MAPK was able to blunt the hypoxia-induced increase in VEGF biosynthesis. These data provide the first direct evidence for a role of p38 MAPK in mediating hypoxia-induced increase in VEGF biosynthesis in human endothelial cells.

Key words: hypoxia; vascular endothelial growth factor; vascular endothelial cells; mitogen-activated protein kinase p38; signal transduction

p38 丝裂原素激活的蛋白激酶在调节低氧诱导人内皮细胞分泌血管内皮生长因子过程中的作用

范 蓓, 王艳霞, 姚 泰, 朱依纯*
复旦大学上海医学院生理与病理生理教研室, 教育部分子医学重点实验室, 上海 200032

摘 要: 血管内皮细胞中血管内皮生长因子(vascular endothelial growth factor, VEGF)的合成增加在促进血管新生的过程中起着非常重要的作用。然而低氧诱导 VEGF 分泌的细胞内信号转导机制还不是很清楚。人脐静脉内皮细胞系(ECV304)在低氧或常氧的状态下培养12~24 h 后分别用实时定量 PCR 和 Western blot 的方法来检测 VEGF mRNA 的表达及ERK1/2 和p38 激酶的磷酸化水平，分泌到培养液中的 VEGF 蛋白用酶联免疫吸附(ELISA)的方法来检测。业已报道，ERK 的抑制剂PD98059 能够抑制低氧诱导的 VEGF 基因的表达，根据这个报道，我们发现在低氧情况下，ECV304 细胞的 ERK1/2 磷酸化水平增高以及 VEGF 的合成增加等这些变化也能被 PD98059 所抑制。本次实验的新发现是 p38 激酶的激酶在低氧诱导 VEGF 合成增加中的作用。p38 激酶的抑制剂 SB202190 能抑制低氧诱导的 VEGF 合成增加，这些数据首次直接证实了 p38 激酶在低氧诱导人内皮细胞分泌 VEGF 增加过程中的作用。

关键词: 低氧; 血管内皮生长因子; 血管内皮细胞; p38 丝裂原素激活的蛋白激酶; 信号转导

中图分类号: R33

Vascular endothelial growth factor (VEGF) is an angiogenic factor playing a pivotal role in most steps of the angiogenic process, e.g., proliferation and migration of endothelial cells, microvascular tube formation and degra-
ulation of extracellular matrix to allow the sprouting of a new microvessel [1-5]. Expression of VEGF has been reported to be regulated in the cardiovascular system under various pathological and physiological conditions such as hypertension [60], ischemia [7], cardiac remodeling [8], hypoxia [9], and organ development [10-11]. Hypoxia-induced angiogenesis has been viewed as a compensatory mechanism in ischemic disorders of the cardiovascular system such as coronary artery diseases [12], among which hypoxia-induced VEGF release from the vascular endothelial cells has been reported to play an obligatory role in promoting the process of angiogenesis in paracrine and/or autocrine pattern [13-16]. The expression level of the VEGF gene is very low in most cells under normoxic conditions and can be up-regulated in hypoxia. It has been reported that hypoxia might induce an increase in biosynthesis of VEGF in cardiac myocytes [17], retinal glial cells [18], vascular smooth muscle cells [19], epithelial cells [20], endothelial cells [21] and many carcinoma cell lines [16-21]. The transcriptional rate of VEGF mRNA as assessed by nuclear run-off transcription assay has been reported to be increased by 2-3 fold during hypoxia [20,22]. However, the underlying mechanisms of hypoxia-induced increase in VEGF biosynthesis remain largely uncertain. Mitogen-activated protein kinase (MAPK) phosphorylation seems to be involved in the biosynthesis of VEGF in hypoxia since kinase inhibitors are able to blunt VEGF release in response to hypoxia [23]. In mammalian cells, the responses to external stress are regulated through three major MAPK signaling pathways, i.e., the extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) [24,25]. The hypoxia-induced activation of the pathways of ERK and JNK has been observed in Hela cells [26], human microvascular endothelial cells-1 [27], and Hep3B cells [28]. However, the role of p38 in mediating the cellular response to hypoxia remains to be investigated. In the present study, we provide direct evidence for suggesting the mediation of p38 in hypoxia-induced VEGF biosynthesis in human umbilical vein endothelial cell lines (ECV304).

1 MATERIALS AND METHODS

1.1 Experimental protocol

Human umbilical vein endothelial cell lines (ECV304) were cultured in normoxic or hypoxic conditions for 12–24 h and harvested for determination of VEGF mRNA expression and phosphorylation of ERK1/2 and p38 MAPK by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis, respectively. Culture medium was collected for the measurement of secreted VEGF protein by enzyme-linked immunosorbent assay (ELISA). To examine the role of ERK1/2 and p38 MAPK in hypoxia-induced VEGF biosynthesis, the cells were treated differently in the following groups: normoxia control (cultured in normoxic condition, treated with vehicle), normoxia + PD98059 (cultured in normoxic condition, treated with 50 µmol/L PD98059, an inhibitor of ERK1/2 phosphorylation [29]), normoxia + SB202190 (cultured in normoxic condition, treated with 20 µmol/L SB202190, an inhibitor of p38 MAPK phosphorylation [30]), hypoxia (cultured in hypoxic condition, treated with vehicle), and hypoxia + PD98059 (cultured in hypoxic condition, treated with 50 µmol/L PD98059), and hypoxia + SB202190 (cultured in hypoxic condition, treated with 20 µmol/L SB202190).

1.2 Cell culture

ECV304 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco Carlsbad, CA, USA) containing 10% calf serum, 100 U of penicillin, 100 µg/ml streptomycin at 37°C in normoxic conditions (21% O2, 5% CO2). Cells were subcultured by disaggregation with trypsin (0.125%)-EDTA (0.01%) in phosphate-buffered saline (PBS) at pH 7.5. In some experiments, serum-free medium (1:1 mixture of Ham’s F12 and DMEM) was added to the cells for 24 h to arrest the growth of the cells. Exposure to hypoxia was achieved by culturing the cells in a humidified airtight chambers which were gassed with 21% O2 or 3% O2 with 5% CO2 for 5 min before the chambers were sealed. The chambers were maintained in an incubator at 37°C for 12 to 24 h and found a variation less than 2% in O2 concentration.

1.3 Western blot analysis

Protein was extracted from the cultured ECV304 cells according to the methods described by Mohamed et al. [31]. All samples were normalized according to protein concentration and separated in 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes (Gelman-Pull, Ann Arbor, MI, USA) using the wet transfer blotting system (Bio-Rad, Hercules, CA, USA). After blocking in Tris-buffered saline (TBS: 120 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4) containing 0.05% Tween 20 and 5% dried milk, the blot was probed with suitable antibodies and incubated overnight at 4°C. The antibodies (1:1 000) for phospho-specific p44/p42 MAPK, total p44/p42 MAPK, phospho-specific p38 MAPK, and total p38 MAPK (Cell Signaling, Beverly MA, USA) were used to detect phospho-ERK, total ERK, phospho-p38 MAPK, and p38 MAPK, respectively. The bands were visualized by chemiluminescence and the intensity of bands was quantified by densitometry.
respectively. Subsequently, the membranes were washed extensively and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1 000) for 2 h at room temperature, washed with TBS containing 0.05% Tween-20 three times, followed by chemiluminescent detection (Pierce Rockford, IL, USA).

1.4 Determination of VEGF by ELISA
ELISA was performed to quanitate VEGF protein released from the cultured cells. The cells (3×10^5) were plated in 50 cm^2 flasks and allowed to grow to 80% confluence. Then, the cells were treated according to the experimental protocols in serum-free medium. The culture medium was taken for VEGF assay using a commercially available kit (Quantibine, R&D system, Minneapolis, MN, USA) according to the protocols provided by the manufacturer. VEGF protein levels were represented as picograms of VEGF per micrograms of total medium protein.

1.5 RNA extraction and real-time RT-PCR analysis
Total RNA was extracted from the cultured cells, quantified by absorbance at 260 nm, normalized, and reverse-transcribed into first-strand complementary DNA (cDNA). 2 µg cDNA mixture obtained from the reverse transcription reaction was used in PCR with a total volume of 50 µl that contain 5 µl 10×PCR buffer, 4.0 µmol/L MgCl_2, 0.2 µmol/L of each primer, 1 µl 10 mmol/L dNTP, and 0.1 µmol/L probe. VEGF mRNA expression was determined with quantitative real-time PCR using iCycler iQ™ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). To minimize sample variations, mRNA expression of the target gene was normalized relative to the expression of the housekeeping gene GAPDH. The probes for the target genes were labeled with 6-carboxy-fluorescein (FAM) reporter dye at the 5’ end and 6-carboxy-tetramethyl-rhodamine (TAMRA) dye as the quencher at the 3’ end. For the VEGF gene, the forward primer was 5’-ATGAACTTTCTGCTGTCTTGGGT-3’, the reverse primer was 5’-ATGATTCTGCCCCCTCTCTTCT-3’ and the probe was FAM-5’-CCTTGCCTTGCTGTACCTCACCA-3’-TAMRA, corresponding to the positions 1039, 1149, and 1071, respectively, of the published sequence (GenBank accession No. BC 023632) [33]. Three-step real-time PCR of denaturing, annealing, and extension was proceeded for 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C for VEGF and GAPDH. Increasing curves of reporter dye fluorescence emission were recorded and analyzed with the iCycler iQ™ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) to determine the threshold cycle (Ct) value. Each sample was run and analyzed in triplicate and the Ct value for VEGF was subtracted from the C t value of GAPDH to yield a ΔCt value. The average ΔCt was calculated for the control group and this value was subtracted from the ΔCt of all other samples (including the control group). This resulted in a ΔΔCt value for all samples which was then used to calculate the fold-induction of VEGF mRNA expression using the formula 2^ΔΔCt, as recommended by the manufacturer (Bio-Rad, Hercules, CA, USA).

1.6 Statistical analysis
Quantitative data were expressed as mean ± SEM. The data were analyzed by a one-way analysis of variance (ANOVA) followed by a Tukey test. In all cases, probability level<0.05 (P<0.05) was taken to indicate statistical difference.

2 RESULTS

2.1 Hypoxia-induced increase in VEGF mRNA expression and secreted VEGF protein
The average ΔCt values of VEGF mRNA for the normoxia control, 12 h hypoxia, and 24 h hypoxia groups were 9.50 cycles, 7.60 cycles, and 10.33 cycles, respectively (∆Ct=3 in each group). The average fold-inductions for these groups were: 1.07 ± 0.12 in normoxia control, 4.05 ± 0.87 in 12 h hypoxia and 0.63 ± 0.20 in 24 h hypoxia (Fig. 1). Analysis of these data showed that 12 h hypoxia induced an increase in VEGF mRNA expression in ECV304 cells (∆Ct=3, P<0.05 versus normoxia control). A significant increase in secreted VEGF protein was observed in the 24 h hypoxia group compared with the normoxia control (P<0.05) (Fig. 2).

2.2 Hypoxia-induced increase in the phosphorylation of ERK1/2 and p38 MAPK
Quantitative immunoblot analysis was performed by normalization of the phosphorylated ERK1/2 and p38 MAPK to the total forms of ERK1/2 and p38 MAPK, respectively, and then expressed as relative fold of their control. The values for the hypoxia, hypoxia + PD98059, and normoxia + PD98059 groups were 12.72 ± 1.94, 1.90 ± 0.26, and
0.44 ± 0.27, respectively (Fig. 3, n=3 in each group). In the experiments measuring p38 MAPK phosphorylation, the values for the hypoxia, hypoxia + SB202190, and normoxia + SB202190 groups were (2.06 ± 0.20), (0.37 ± 0.04), and (0.06 ± 0.02), respectively (Fig. 4, n=3 in each group). Twenty-four hours of hypoxia caused a marked increase in the phosphorylation of both ERK1/2 and p38 MAPK which was blunted by PD98059 and SB202190, respectively (Fig. 3, 4).

3.3 Effect of p38 MAPK inhibition on hypoxia-induced increase in VEGF mRNA expression and secreted VEGF protein

VEGF mRNA expression was measured by real-time RT-PCR. The average fold-inductions of VEGF mRNA measured by real-time RT-PCR for the normoxia control, normoxia + SB202190, hypoxia, and hypoxia + SB202190 groups were 1.02 ± 0.14, 1.15 ± 0.54, 3.64 ± 0.56, and 0.91 ± 0.32, respectively (Fig. 5, n=3 in each group). The percentage values of secreted VEGF levels over the normoxia control group for the normoxia + SB202190, hypoxia + PD, hypoxia + PD98059 group, PD, normoxia + PD98059 group, n=3 in each group. Fold over control means fold of control’s density. *P<0.05 vs control.

Fig. 1. Twelve hours of hypoxia induced a significant increase in VEGF mRNA level in ECV304 cells determined by real-time RT-PCR. All measurements were performed in triplicate. Control, normoxia control group; 12 h, 12 h hypoxia group; 24 h, 24 h hypoxia group. Each column represents mean ± SEM, n=3. *P<0.05 vs control.

Fig. 2. Hypoxia-induced a significant increase in secreted VEGF protein levels in the culture medium of ECV304 cells determined by ELISA. All measurements were performed in duplicate. Control, normoxia control group; 12 h, 12 h hypoxia group; 24 h, 24 h hypoxia group. Each column represents mean ± SEM, n=3. *P<0.05 vs control.

Fig. 3. Twenty four hours of hypoxia induced a marked increase in the phosphorylation of ERK1/2 which was blunted by PD98059. A: Graph representation of the values of mean ± SEM. B: Original figures of Western blot analysis for ERKs. Control, normoxia control group; Hypoxia, hypoxia group; Hypoxia + PD, hypoxia + PD98059 group; PD, normoxia + PD98059 group. n=3 in each group. Fold over control means folds of control’s density. *P<0.05 vs control.
3 DISCUSSION

ECV304 is characterized by a cobblestone monolayer growth pattern, high proliferative potential without any specific growth factor requirement, and anchorage dependency with contact inhibition. Ultrastructurally, endothelium-specific Weibel-Palade bodies were identified. Although one of the endothelial cell markers, Factor VIII-related antigen (VIIIIR:Ag) was negative in this cell line, immunocytochemical staining for the lectin Ulex europaeus I (UEA-I), and PHM5 (anti-human endothelium as well as glomerular epithelium monoclonal antibody) was positive, and angiotensin-converting enzyme (ACE) activity was also demonstrated. In addition, ECV304 displayed negativity for alkaline and acid phosphatase and for the epithelial marker keratin. All of these findings suggest that ECV304 cells originated from umbilical vein endothelial cells by spontaneous transformation. It has been used as a model system for endothelial cells in vitro to study angiogenesis, cell migration, cytokine expression, signal transduction of VEGF, permeability of monolayers, receptor pharmacology, calcium channel, endothelial expression of MM-9 (matrix metalloproteinase-9), the source of complement proteins from endothelial cells and so on [34]. VEGF is a potent mitogen specific for endothelial cells. Its expression is dramatically induced by low oxygen tension in a variety of conditions. The role of p38 MAPK in hypoxia-induced VEGF release was studied in ECV304 cells.

Fig. 4. Twenty four hours of hypoxia induced a marked increase in the phosphorylation of p38 MAPK which was blunted by SB202190. A: Graph representation of the values of mean ± SEM. B: Original figures of Western blot analysis for p38 MAPKs. Control, normoxia control group; Hypoxia, hypoxia group; Hypoxia + SB, hypoxia + SB202190 group; SB, normoxia + SB202190 group. n=3 in each group. Fold over control means folds of control’s density. *P<0.05 vs control.

Fig. 5. Inhibition of p38 MAPK phosphorylation with SB202190 blunted the hypoxia-induced increase in VEGF mRNA expression as measured by real-time PCR. All measurements were performed in triplicate. Control, normoxia control group; SB, normoxia + SB202190 group; Hypoxia, hypoxia group; Hypoxia + SB, hypoxia + SB202190 group. Each column represents mean ± SEM, n=3. *P<0.05 vs control.

Fig. 6. Treatment with SB202190 blunted the hypoxia-induced increase in secreted VEGF protein levels in the culture medium of ECV304 cells as measured by ELISA. All measurements were performed in duplicate. Control, normoxia control group; SB, normoxia + SB202190 group; Hypoxia, hypoxia group; Hypoxia + SB, hypoxia + SB202190 group. Each column represents mean ± SEM, n=3. *P<0.05 vs control.
cell types, and it has been suggested to be a key mediator of hypoxia-induced angiogenesis. Although VEGF action is targeted to endothelial cells, it is generally believed that these cells do not express VEGF. However, the mechanisms by which hypoxia regulates VEGF production remain unclear. It reported that pulmonary artery endothelial cells do not express VEGF under basal conditions; however, significant VEGF mRNA levels accumulate when these cells are exposed to hypoxia. The expression of VEGF by endothelial cells in response to hypoxia may provide an important mechanism by which endothelial cell permeability and proliferation is regulated in an autocrine manner [35].

Hypoxia-induced expression of VEGF gene is regulated at transcriptional, post-transcriptional and translational stages [12,36]. It has been reported that p42/p44 MAPK cascade activates the VEGF promoter in hypoxia [37]. Mukhopadhyay et al. further demonstrated that PD98059, an ERK inhibitor, was able to blunt the hypoxia-induced activation of the expression of VEGF gene [38]. In accordance with this report, we observed an increase in ERK1/2 phosphorylation and VEGF biosynthesis in ECV304 cells cultured in hypoxia, and this increase was blocked by PD98059 (Fig. 3 and unpublished data).

The novel finding of the present study is that an activation of p38 MAP kinase is involved in hypoxia-induced increase in VEGF biosynthesis. In the human umbilical vein endothelial cell line ECV304, we observed a hypoxia-induced increase in p38 MAP kinase phosphorylation which was blunted by the p38 MAP kinase inhibitor SB202190. Moreover, treatment with SB202190 blocked the hypoxia-induced increase in VEGF biosynthesis, suggesting a role for p38 MAP kinase in the intracellular signaling systems triggered by hypoxia.

p38 MAPK is an intracellular signaling element downstream of RAS and MKK3,6 (Fig. 7). p38 MAPK has been shown to play a major role in apoptosis, cytokine production, transcriptional regulation, and cytoskeletal reorganization, and has been causally implicated in disease states such as sepsis, ischemic heart disease, arthritis, human immunodeficiency, virus infection, and Alzheimer’s disease [39]. However, a role for p38 MAPK in mediating hypoxia-induced increase in VEGF biosynthesis in endothelial cells has not yet been reported. As illustrated in Figure 7, our present study reveals a role of p38 MAPK in hy-

---

**Fig. 7.** Multiple regulatory pathways of VEGF expression in hypoxia. Schematic illustration of intracellular signaling mechanisms of hypoxia-induced VEGF biosynthesis. NADPH-ox, Nicotinamide adenine dinucleotide phosphate Oxidase; PI-3K, PI-3-kinase; PIP3, phosphatidylinositol 3,4,5-triphosphate 3,4,5; ROS, reactive oxygen species; ERK1/2, extracellular signal regulated kinase 1/2; VEGF, vascular endothelial growth factor; MEK, mitogen-activated protein kinase kinase; MEKK, MEK kinase; MKK, MAPK kinase; JNK, c-Jun NH2-terminal kinase; HIF-1, hypoxia-inducible factor 1.
hypoxia-induced increase in VEGF biosynthesis. The substrates of p38 MAPK are usually either transcription factors or other protein kinases, such as C/EBP Homologous Protein (CHOP), a member of the C/EBP family of transcription factors, the ternary complex factor (TCF) Sapp1a, AFT-2, and ATF-6. MAPKAP kinase-2, mitogen and stress-activated protein kinase (MSK)-1 and MSK2, and MAPKAP kinase-3 are also activated by p38 MAPK [29]. Nevertheless, the downstream molecular mechanisms of p38 MAPK activation in hypoxia-induced VEGF release, such as activation of transcription factors or other protein kinases, remain to be further clarified. The interaction between p38 MAPK and hypoxia-inducible factor 1 (HIF-1) may also be a point of potential interest.

In addition to the intracellular signaling pathways mentioned above, one more question in pendency is how hypoxia initiate the intracellular signaling cascades, i.e., how the cells monitor oxygen content and trigger the cellular responses to hypoxia. It is not certain that whether multiple kinds of oxygen sensors exist in the same cell, or different mechanisms of oxygen sensing occur in different cells, or a single kind of oxygen sensor may regulate diverse responses to hypoxia in mammalian cells.

In conclusion, our present study shows that both p38 MAPK and ERK1/2 activation are involved in hypoxia-induced increase in VEGF biosynthesis in the human umbilical vein endothelial cell lines ECV304.

REFERENCES


28 Hur E, Chang KY, Lee E, Lee SK, Park H. Mitogen-activated protein kinase inhibitor PD98059 blocks the trans-activation but not the stabilization or DNA binding ability of hypoxia-inducible factor-1alpha. Mol Pharmacol 2001; 59: 1216-1224.


