Acta Physiologica Sinica, April 25, 2006, 58 (2): 124-128
http://www.actaps.com.cn

Research Paper

Effects of basic fibroblast growth factor on the expressions of angiogenic gene profile and cyclooxygenase-2 in brain microvascular endothelial cells

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Abstract: The present study aimed to investigate the effects of basic fibroblast growth factor (bFGF) on the expressions of angiogenesis-related genes in a mouse brain microvascular endothelial cell line, namely bEnd.3, using cDNA microarray. The effects of bFGF (10 ng/ml) on mRNA and protein expressions of cyclooxygenase-2 (COX-2), an angiogenesis bystander molecule, were further investigated. cDNA microarray was employed to study the effects of bFGF on the expressions of angiogenic genes in a high throughput pattern. RT-PCR was used to study the effect of bFGF on COX-2 mRNA expression. Western blot and immunocytochemistry were utilized to study the effect of bFGF on COX-2 protein expression. The results showed that, 2 h after bFGF treatment, pro-angiogenic genes (Adamts1, MMP-9, Ang-1, PDGF B, G-CSF, FGF16, IGF-1, etc.) were significantly upregulated, whereas anti-angiogenic genes (TIMP-2, TSP-3, etc.) were significantly downregulated. The bystander molecule in angiogenic pathway COX-2 mRNA and protein expressions were significantly upregulated after bFGF treatment. It is suggested that triggering angiogensis switch through upregulating pro-angiogenic gene and downregulating anti-angiogenic gene expression is one of the major mechanisms of bFGF-induced angiogenesis. The expression change of COX-2, as a bystander molecule, was observed after bFGF treatment in bEnd.3 cells and the significance was discussed.

Key words: basic fibroblast growth factor; microvascular endothelial cell; angiogenesis; cyclooxygenase

碱性成纤维细胞生长因子对脑微血管内皮细胞血管新生基因谱和环加氧酶-2表达的影响

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摘 要：本文研究了碱性成纤维细胞生长因子(basic fibroblast growth factor, bFGF)对小鼠脑微血管内皮细胞(microvascular endothelial cell, MVEC)株bEnd.3中血管新生相关基因表达谱的改变，并重点从mRNA、蛋白质和细胞水平检测bFGF对血管新生旁观分子环加氧酶-2 (cyclooxygenase-2, COX-2)表达的影响。用特异性小鼠血管新生基因芯片高通量检测bEnd.3细胞基因谱表达的改变，分析促血管新生基因及抑制血管新生的基因表达谱的变化；用RT-PCR、Western blot、免疫细胞化学等方法分别从mRNA、蛋白质和细胞水平检测COX-2表达变化及细胞内的定位。结果发现用10 ng/ml的bFGF刺激bEnd.3细胞2 h后多种促血管新生基因表达明显上调，如Adamts1、MMP-9、Ang-1、PDGF B、G-CSF、FGF16、IGF-1等分别上调3、8、120、5.2、4.5、1.7、2.7倍。与此同时，多种抑制血管新生的基因表达相应下调，如TSP-3、TIMP-2、TGFβ1等表达分别下调3.4、1.5和3.5倍。RT-PCR和Western blot的结果证实，bFGF可以上调COX-2 mRNA的表达和蛋白质的合成。免疫组化的结果表明，COX-2主要分布在胞浆。以上结果提示：bFGF具有上调促血管新生基因表达,下调抑制血管新生基因表达的作用，两者协同作用，促进血管新生。同时bFGF还可以明显促进血管新生旁观分子COX-2 mRNA的表达和蛋白质的合成。本文讨论了bFGF引起MVEC内COX-2表达上调的意义。

关键词：碱性成纤维细胞生长因子；微血管内皮细胞；血管新生；环加氧酶

中图分类号：Q463; R331.3+5

Received 2005-09-27 Accepted 2005-12-28

This work was supported by the Key Department Development Fund of "211" Project, Ministry of Education of China.

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Angiogenesis refers to the formation of new capillary networks located at sites of previous blood vessels in a gemmate or ablastemic manner through migration and proliferation of endothelial cells (ECs) to form blood capillary and /or venula under physiological or pathological conditions[1,2]. After the initiation of angiogenesis, ECs first dissociate from perivascular cells and the extracellular matrix (ECM) is degraded by protease; then ECs, in particular microvascular endothelial cells (MVECs), migrate, proliferate and differentiate to form tube-like structure. ECs are recombined with pericytes and ECM is remodeled, finally resulting in the formation of functional capillary networks. Angiogenesis is involved in many physiological and pathological processes such as endometrial cycle, trauma renovation and healing. Additionally, angiogenesis plays an important role in the initiation, promotion and metastasis of tumors, rheumatoid arthritis and diabetic retinopathy, and so on[3]. Angiogenesis is precisely regulated by many pro-angiogenic factors and anti-angiogenic factors[4]. Basic fibroblast growth factor (bFGF), as a pro-angiogenic factor, is able to exert direct or indirect effects on every aspect of the process of angiogenesis. Previous studies demonstrated that bFGF may induce the migration, proliferation, and differentiation of ECs. bFGF-induced migration and proliferation of ECs are differentiated into new vascular structures. bFGF also regulates the activities of extracellular molecules including collagenase, proteins, urokinase-type plasminogen activator (uPA), and integrins to form new capillary cord structures. During this process, a lot of genes, including pro-angiogenic genes and anti-angiogenic genes, are involved. But so far little is known about the general changes of these genes after application of bFGF in MVECs[5]. In the present study, we investigated the effects of bFGF on angiogenesis-related gene expression in mouse brain MVECs by cDNA microarray, especially focusing on the influence of bFGF on cyclooxygenase-2 (COX-2) expression at mRNA and protein level, to further elucidate the mechanism underlying bFGF-promoted angiogenesis.

1 MATERIALS AND METHODS

1.1 *Apparatus and materials*

The inverted microscope was purchased from Leica Company. The cell culture incubator and the superclean bench were purchased from Heraeus Company and Suzhou Purification Equipment Co., Ltd., respectively.

1.2 *Reagents*

High glucose DMEM medium was from Gibco Company and fetal bovine serum was from Hangzhou Sijiqing Biological Engineering Material Co., Ltd. bFGF and trypsase as well as EDTA were purchased from PeproTech Company and Ameresco Company, respectively. GEArray™ series mouse angiogenesis gene microarray was product of SuperArray Company (USA). Rabbit anti mouse COX-2 polyclonal antibody and HRP-conjugated goat anti rabbit IgG antibody were from Santa Cruz Company. The kit for reverse transcription, PCR Master Mix and DNA marker were purchased from MBI Company. BCA-100 protein quantification kit and SuperSignal® West Pico Chemiluminescent Substrate were from Shanghai Shengnengbocai Biological Technology Co. Ltd. and Pierce Chemical Company, respectively. ABC kit and DAB kit were purchased from Sino-American Biotechnology Company. Other reagents met national analytical pure standards.

1.3 *Culture of bEnd.3 cells*

Strain bEnd.3 was from ATCC and was confirmed to be MVEC by us[6]. The cells were cultured at 37 ºC in 5% CO_2 with high glucose DMEM medium supplemented with 10% fetal bovine serum. Every 2 to 3 d, the medium was renewed.

1.4 *Serum-free medium treatment and starvation*

After bEnd.3 cells grew to about 80% (cell total number is 5×10^6) high glucose DMEM medium supplemented with 10% fetal bovine serum was replaced by free-serum high glucose DMEM medium supplemented with 0.1% BSA. After starvation with free-serum high glucose DMEM medium for 12 h, cells were subjected to the treatment.

1.5 *Mouse angiogenesis gene microarray analysis*

RNA was extracted from bEnd.3 cells using TRIzol method as described previously and then the cDNA was reversely transcripted to cDNA. Followed protocol provided by the gene microarray kit, the detection of mouse angiogenesis gene microarray, was carried out twice. Every gene was measured four times and the average value was adopted. The results were analyzed with the specific software provided by SuperArray Company. The cells without treatment of bFGF were regarded as the control.

1.6 *RT-PCR of COX-2*

The total RNA was extracted from bEnd.3 cells according to the manufacturer's instruction and then the cDNA was synthesized through using reverse transcriptase. The sequence of COX-2 upstream primer is 5'-TTGAGGA GAGCAGATGGGTT-3' and the downstream primer is 5'-GCTTCGGGAGCACAACAA-3'. GAPDH was used as an internal control, and its upstream and downstream primers are 5'-AAGCCGATCTTCTTGTGCAGTG-3' and 5'-

GGCCTTGACTGTGCCGTTGAATTT-3', respectively. The reaction started with an denaturation time of 5 min at 94 ºC, followed by 25 cycles with each cycle consisting of 1 min at 94 ºC, 1 min at 55 ºC, 1 min at 72 ºC, at last extended at 72 ºC for 1 min. PCR products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide (EB) and photographed for further study.

1.7 *Western blot of COX-2*

The total protein of bEnd.3 cells was extracted and its concentration was measured using BCA methods. Protein was subjected to SDS-PAGE gel for electrophoresis and then transferred to PVDF membrane. The membrane was blocked for 2 h with TBST blocking solution containing 5% skimmed milk in TBST, and incubated with rabbit anti-COX-2 mouse polyclonal antibody at 4 ºC over one night and then HRP-conjugated goat anti rabbit IgG antibody (1: 3000 dilution) for 1 h at room temperature followed by ECL visualization of the bands.

1.8 *Immunocytochemical staining of COX-2*

The first antibody and second antibody were diluted to 1: 100 and 1:200, respectively. The dilution liquid was used to be the negative control. Immunocytochemical staining of COX-2 was performed according to the manufacturer's instruction.

2 RESULTS

2.1 Effects of bFGF on gene expression profiles

Based on our previous studies[7], we adopted 10 ng/ml bFGF as an effective dose. After treatment of 10 ng/ml of bFGF for 2 h, genes in bEnd.3 cells were divided into five groups according to the rate of their expression intensity (the intensity of genes in the treated cells/ the intensity of gene expression in the control cells): significantly upregulated genes, the ratio more than 1.5; slightly upregulated genes, the ratio between 1 and 1.5; unchanged genes, the ratio is 1; slightly downregulated genes, the ratio between 0.5 and 1; significantly downregulated genes, the ratio less than 0.5. The detailed results were shown in table 1 and Fig.1.

2.2 Effects of bFGF on COX-2 mRNA expression

The expression of COX-2 mRNA was significantly increased after treatment with 10 ng/ml bFGF for 2 h but the expression of GAPDH mRNA had no obvious change. The detailed results were shown in Fig.2.

2.3 Effects of bFGF on COX-2 protein production in bEnd.3 cells

The production of COX-2 protein in bEnd.3 cells was significantly increased after treatment with 10 ng/ml bFGF for 8 h but actin had no obvious changes (Fig.3, 4).

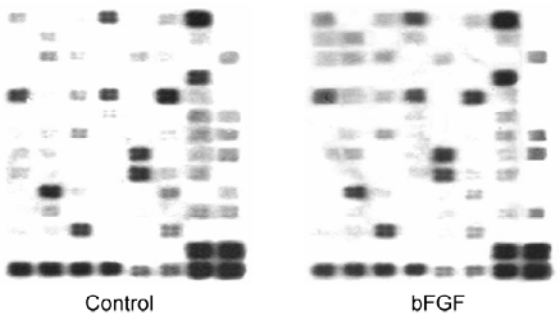

Fig. 1. Effects of bFGF on the expressions of angiogenic genes.

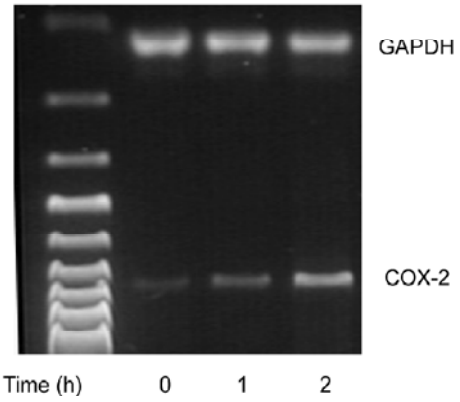

Fig. 2. Effects of bFGF (10 ng/ml) on COX-2 mRNA expression in bEnd.3 cells.

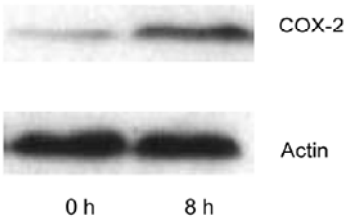

Fig. 3. Effects of bFGF (10 ng/ml) on COX-2 protein expression in bEnd.3 cells (Western blot).

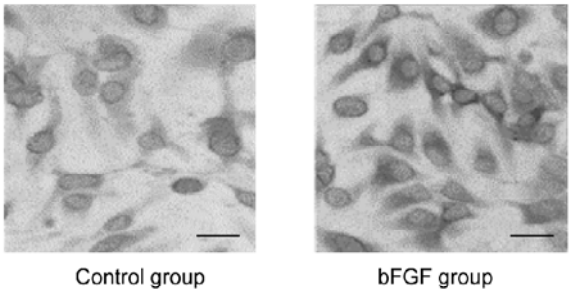

Fig. 4. Effects of bFGF (10 ng/ml) on COX-2 protein expression in bEnd.3 cells (immunocytochemistry). Scale bar, 10 μm.

Table 1. Effects of bFGF on the expressions of genes that related to angiogenesis in bEnd.3 cells and their grouping

Group	Gene names
Obviously up-expressing genes	Adamts 1, Angiopoietin-1, CD36, G-CSF, Ephrin B2, Ephrin B4, c-ets1, FGF16, FGFR3, FGFR4, VEGFR, Fn1, IFN-β1, IGF-1, TSP-2, TIMP2, VCAM-1, FGF16, MMP-9, Neuropilin, PDGF B, PLAU, COX-2, Procollagen (type XVIII, α1)
Slightly up-expressing genes	PECAM1, Scya2, Tie1, TIMP1, Tenascin C, β-actin
Non-expressing genes	β-glycan, VEGF-B, VEGF-C, pUC18, TGFβ2, TNFα, IFNα1, PEDF, TGFβ3, TGFβR1 (ALK-5), Integrin αV, TSP-1, GAPDH, COX-1
Slightly down-expressing genes	H1α, ID1, Integrin 5, CD61, NOS3, VEGF, PDGF A, PDGFRα, PGF, PAI-1, SPARC, RPL13A, Cyclophlin A
Obviously down-expressing genes	SR-A, Maspin, TGFβ1, TGFβR2, TIMP2, TSP-3

3 DISCUSSION

The precise role and regulatory mechanism of bFGF, as a strong pro-angiogenic factor, on angiogenesis hasn't been fully elucidated yet. Current studies have confirmed that multiple genes were involved in angiogenesis regulation. But the study on changes of angiogenic gene profile in this field is less. Gene microarray has advantages in high-throughput screening of gene expression change. In present study, we detected the expression changes of angiogenesis-related gene profile in bEnd.3 cells after bFGF treatment by using a specific angiogenesis gene microarray.

In this study we used serum-free medium treatment to avoid interferences of endogenous growth factors in serum. Our previous studies have demonstrated that 10 ng/ml bFGF could engender obvious biological effects on ECs[7]. The results of gene microarray analysis showed upregulation of some pro-angiogenic genes in bEnd.3 cells after 10 ng/ml bFGF treatment for 2 h. The angiopoietin-1, PDGF B, G-CSF, IGF-1 gene expressions were upregulated 120, 5.2, 4.5 and 2.7 times, respectively. They possess stimulative effects on the angiogenesis directly. The expressions of the related receptors such as VEGFR, FGFR3 and FGFR4 were also upregulated 7.4, 2.2 and 1.6 times, respectively. The upregulation of FGFR can lead to enhanced effects of bFGF. The expressions of Adamts 1, MMP9 were upregulated 3 times and 8 times, respectively. Both Adamts 1 and MMP-9 are proteases that can degrade ECM. At the same time other genes were downregulation, for example TIMP-2. There was a 1 600 times downregulation of TIMP-2 and their relative expression intensity was only 5.4×10^{-5}. TIMP-2 can inhibit various MMP activities. So their combined changes stimulate further degradation of ECM which is one of the most important facts inducing angiogenesis[8].

Our previous studies in cultured ECs demonstrated that bFGF may induce an effect on production of VEGF indirectely[7]. In this research we found that after treatment of bFGF (10 ng/ml) the expression of VEGFR was upregulated 7.5 times, however, VEGF was downregulated only 50%, so the expression of VEGF was still high, indicating the total effect of VEGF was increased for promoting angiogenesis. The expressions of Ephrin B2 and Ephrin B4 involving in arteriovenous differentiation[9] were increased 2.2 and 6.5 times, respectively. Correspondingly, some anti-angiogenic factor genes such as TSP-3, TIMP-2, were downregulated (3.4, 1.5 and 3.5 times, respectively). Breifly, the major changes in bEnd.3 cells induced by bFGF are upregulation of some pro-angiogenic genes and downregulation of some anti-angiogenic genes. Their coordination effect is stimulation of angiogenesis.

Recent studies proved that there is a kind of angiogenesis bystander molecules, including COX-2, which are involved in the regulation of angiogenesis[10]. They take a role in angiogenesis after exposure to some stimuli such as growth factors. The results of gene microarray showed that it could increase 6 times of COX-2 expression after treatment of bFGF in bEnd.3 cells. COX-2 gene has response elements of many growth factors in 5' terminal so that many growth factors can regulate its expression.

Thereby COX-2 gene is an immediate-early gene[11]. Some researchers have found that many growth factors including bFGF can induce COX-2 expression. Using RT-PCR and gene microarray method, we further confirmed bFGF may induce COX-2 expression. Furthermore, our research found that bFGF could promote synthesis of COX-2 protein and COX-2 was located in cytoplasm. COX-2 subsequently may stimulate angiogenesis. The metabolic products of COX-2 cascade also confer effects on angiogenesis including the migration of ECs and formation of tube-like structure[12]. Furthermore, the upregulation of COX-2 plays an important role in angiogenesis in the bFGF-induced *in vivo* angiogensis model, which is blocked by the specific inhibitors of COX-2 such as NS-398[13].

Our study and other investigators have demonstrated that bFGF can increase synthesis of COX-2 protein of ECs from various tissues, however, different ECs might not elucidate the real effects of bFGF on brain MVECs[14], because ECs, especially MVECs have tissue heterogenicity. Our study showed that bFGF could upregulated COX-2 expression of brain MVECs at mRNA and protein level. MVECs of brain play an important role in regulating brain functions in physiological and pathological conditions. When cerebral vessels are injured to release many active factors including bFGF, the relased bFGF can protect neurons and neuroepithelial cells against injury, which has been accepted by many scholars and doctors. However, with respect to other effects bFGF induced, such as enhanced secretion of prostaglandin-like active factors induced by regulated expression of COX-2, on injury of neurons and neuroepiphilial cells have not been paid enough attention[15,16] and need further investigation.

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