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Histamine Synergistically Promotes bFGF-Induced Angiogenesis by Enhancing VEGF Production via H1 Receptor

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ABSTRACT

Histamine, a major mediator present in mast cells that is released into the extracellular milieu upon degranulation, is well known to possess a wide range of biological activities in several classic physiological and pathological processes. However, whether and how it participates in angiogenesis remains obscure. In the present study, we observed its direct and synergistic action with basic fibroblast growth factor (bFGF), an important inducer of angiogenesis, on in vitro angiogenesis models of endothelial cells. Data showed that histamine (0.1, 1, 10 μM) itself was absent of direct effects on the processes of angiogenesis, including the proliferation, migration, and tube formation of endothelial cells. Nevertheless, it could concentration-dependently enhance bFGF-induced angiogenesis as well as production of vascular endothelial growth factor (VEGF) from endothelial cells. The synergistic effect of histamine on VEGF production could be reversed by pretreatments with diphenhydramine (H1-receptor antagonist), SB203580 (selective p38 mitogen-activated protein kinase (MAPK) inhibitor) and L-NAME (nitric oxide synthase (NOS) inhibitor), but not with cimetidine (H2-receptor antagonist) and indomethacin (cyclooxygenase (COX) inhibitor). Moreover, histamine could augment bFGF-incuced phosphorylation and degradation of IκBα, a key factor accounting for the activation and translocation of nuclear factor κB (NF-κB) in endothelial cells. These findings indicated that histamine was able to synergistically augment bFGF-induced angiogenesis, and this action was linked to VEGF production through H1-receptor and the activation of endothelial nitric oxide synthase (eNOS), p38 MAPK, and IκBα in endothelial cells. J. Cell. Biochem. 114: 1009–1019, 2013.

KEY WORDS: HISTAMINE; BASIC FIBROBLAST GROWTH FACTOR; ANGIOGENESIS; SYNERGISTIC ACTION

Angiogenesis is the formation of new capillaries from preexisting blood vessels. It takes place in a variety of physiological settings including embryonic development, wound healing and tissue regeneration [Pap and Distler, 2005], and is also closely related to the pathogenesis of many diseases such as cancer, cardiovascular diseases, diabetic retinopathy and rheumatic arthritis. The process of angiogenesis is subject to the regulation of multiple endogenous factors. The pro-angiogenic agents include several growth factors such as fibroblast growth factor (FGF) family and VEGF family, cytokines, chemokines, angiogenin, etc. The anti-angiogenic agents include some gene products such as thrombospondin-1 (TSP-1) and pigment epithelium-derived factor (PEDF) and a large number of protein proteolytic fragments such as angiostatin, endostatin and tumstatin [Bouïs et al., 2006; Sato, 2006].

BFGF is one of most potent and best-characterized pro-angiogenic factors [Arai et al., 2006]. As a prototype member of

Abbreviations used: bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; NOS, nitric oxide synthase; COX, cyclooxygenase; NF-κB, nuclear factor κB; eNOS, endothelial nitric oxide synthase; TLR, toll-like receptor; HUVECs, human umbilical vein endothelial cells; ERK, extracellular regulated protein kinases; JNK, Jun-N-terminal kinase.

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13 structurally related heparin-binding growth factors, bFGF is a mitogen of several types of cells, such as vascular endothelial cells and fibroblasts. In several kinds of tumors, it has been reported that angiogenic switch correlated with the exporting of bFGF from tumor cells [Nugent and Iozzo, 2000]. Furthermore, bFGF could work through augmenting the production of VEGF and in cooperation with other factors and co-activators [Yoshiji et al., 2002; Bao et al., 2005; Jeon et al., 2006].

In addition to the classic pro-angiogenic factors mentioned above, some pro-inflammatory and immunomodulatory factors are also demonstrated their indispensable effectiveness in blood vessel development [Kim et al., 2009; Wu and Zhou, 2009]. Histamine, a mediator mainly secreted by mast cells, has a wide range of biological activities. Its participation in angiogenesis process is increasingly attracting the attention of researchers in recent years [Ghosh et al., 2001; Li et al., 2001, 2003; Talreja et al., 2004; Tan et al., 2007; De Luisi et al., 2009; Kubecova et al., 2011; Kwon et al., 2011]. There was a report that histamine could directly promote new vessel growth in tumor cells via promoting the release of VEGF [Ghosh et al., 2001]. Histamine is able to act on endothelial cells, the key player of angiogenesis, to induce the transient expressions of P-selectin, prostacyclin and platelet-activating factor, and the production of cytokines (such as IL-6 and IL-8) as well as several other inflammation- and angiogenesis-related proteins (including eNOS, COX, and toll-like receptor (TLR) 2 and 4) [Li et al., 2001, 2003; Talreja et al., 2004; Tan et al., 2007]. Furthermore, some antihistaminics were recently reported to have anti-angiogenesis potentials and were proposed to be promising therapeutic agents for angiogenesis-related diseases. Carebastine, the active metabolite of ebastine (a selective second-generation histamine H1-receptor antagonist) was demonstrated to have anti-angiogenic properties as evaluated by both in vitro assays in human umbilical vein endothelial cells (HUVECs) and human pulmonary artery endothelial cells (HPAECs) and in vivo chick embryo chorioallantoic membrane (CAM) assay [De Luisi et al., 2009; Kubecova et al., 2011]. However, the details about how histamine works on endothelial cells remain unclear, and several issues such as whether it directly regulates endothelial cells or functions via cooperating with other pro-angiogenic factors are eager for settling.

The present study was performed to clarify the effects of histamine itself as well as its combination with bFGF on in vitro angiogenesis of HUVECs. The focus was put on the auto-secretion of VEGF and the relative mechanisms.

MATERIALS AND METHODS

REAGENTS AND CHEMICALS

M199, bFGF, trypsin, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY); new-born calf serum (NBCS) was purchased from PAA (Laboratories GmbH, Austria); 3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Tween-20, indomethacin, diphenhydramine hydrochloride, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), dithiotheitol (DTT), phenylmethylsulphonyl fluoride (PMSF), cycloheximide and SB203580 were purchased from Sigma Chemical Co. (St. Louis, MO). Histamine phosphate, IκB-α, p-extracellular regulated protein kinases (ERK), p-p38, p-Jun-N-terminal kinase (JNK), GAPDH monoclonal antibodies, enhanced chemiluminescent (ECL) plus reagent kit and the peroxidase-conjugated secondary antibody were purchased from KangChen Bio-tech (Shanghai, China). ENOS, p-eNOS monoclonal antibodies were purchased from Bioworld Technology Inc. (St. Louis Park, MN). Primers were ordered from SBS Genetech (Beijing, China). The other chemicals and reagents used were of analytical grade.

ISOLATION AND TREATMENT OF HUVECS

Umbilical cords were obtained from Nanjing MaiGaoQiao Hospital (Nanjing, China). Endothelial cells were isolated from the cords as described previously [Hotchkiss et al., 2002]. The harvested cells were seeded into 25 cm^2 flasks precoated with 0.2% (w/v) gelatin (Promega, Madison, WI). Culture medium consisted of M199 supplemented with 20% (v/v) newborn calf serum, 2 mM L-glutamine, 5 U/ml penicillin G, 5 μg/ml streptomycin sulfate and 7.5 μg/ml endothelial cell growth supplement (Sigma). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed after 24 h and every 2 days thereafter until confluence. All experiments were performed using HUVECs at passages 3–5. Trypsin (0.25%) in Dulbecco's phosphate-buffered saline was used to digest and passage.

HUVECs were rendered quiescent for 2 h before exposure to histamine. After incubation with histamine for 24 h, bFGF was added. Tool drugs, including diphenhydramine, cimetidine, indomethacin, cycloheximide, L-NAME, and SB203580, were added 1 h prior to the stimulation of bFGF.

PROLIFERATION ASSAY

The proliferation ability of endothelial cells was determined by MTT assay. HUVECs were plated at a density of 1×10^5 cells/well in 96-well plates and incubated at 37°C in 5% CO_2 incubator overnight. For groups of histamine alone, HUVECs were exposed to various concentrations of histamine (0.1, 1, 10, 100 μM) for 24 h. For groups of histamine plus bFGF, HUVECs were initially pretreated with different concentrations of histamine and 10 ng/ml bFGF for 24 h. After these steps, 20 μl of MTT (5 mg/ml) was added to each well and incubated for an additional 4 h. The supernatant was removed and the formazan crystals were subsequently solved with 150 μl DMSO. The optical absorbance at 540 nm was recorded using a Model 1500 Multiskan spectrum microplate Reader (Thermo, Waltham, MA).

TUBE FORMATION ASSAY

Formation of vascular-like structure by HUVECs was assessed by the method described previously [Kureishi et al., 2000]. Briefly, matrigel was diluted to 4 mg/ml with cold phosphate-buffered saline (PBS) and added to 24-well plates in a total volume of 200 μl each well. Then, the plates were incubated at 37°C for 30 min to allow the gel to polymerize. HUVECs were plated at 2×10^4 cells/cm^2/well onto the culture plates coated with matrigel, and incubated with different concentrations of histamine (0, 0.1, 1, 10 μM) for 24 h. Medium containing 2% NBCS in the absence or presence of bFGF (10 ng/ml) was added and cells were incubated for another 12 h at 37°C. Then, tubular structures were quantified by counting the tube numbers

under microscopy and five randomly chosen fields were analyzed for each well.

RAT AORTIC RING ASSAY

Six-week male Sprague–Dawley (SD) rats weighing 250–300 g were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). The procedures and protocols of the study followed our institutional guidelines and were conducted in accordance with the Animal Care and Use Committee of China Pharmaceutical University. Animals were housed in a room with constant temperature ($23 \pm 2^{\circ}C$) and humidity ($55 \pm 5\%$), exposed to a 12 h light and dark cycle, and fed a standard diet and water ad libitum.

As described previously [Garrabrant et al., 2004], the thoracic aortas obtained from rats were immediately transferred to a culture dish with serum-free medium. Then they were cut into 1 mm long rings and set in 96-well plate pre-coated with 40 μl growth factor-reduced-matrigel after the fibroadipose tissues around the aortas were carefully removed by gentle scraping. Additional matrigel (40 μl) was added into the aortic rings to allow solidify. Serum-free endothelial growth medium (200 μl) supplemented with 200 μg/ml ECGS were added to the wells. Then the microvessel sproutings of aortic rings were observed for one week. For groups of histamine alone, aorta rings were exposed to various concentrations of histamine (0, 0.1, 1, 10, 100 μM) for 4 days; for groups of histamine plus bFGF, aorta rings were initially pretreated with different concentrations of histamine for 24 h and then incubated with 10 ng/ml bFGF for 3 days. The pictures of microvascular outgrowth were photographed in Day 4 under an inverted light microscope.

MEASUREMENT OF VEGF-A PROTEIN

The amount of VEGF-A was analyzed using an ELISA kit according to the manufacturer's recommendations [Bello et al., 2008]. In brief, HUVECs were cultured in 6-well plates for 2 h, incubated in the medium containing 2% NBCS with or without histamine (0.1, 1, 10 μM) for 24 h, and then simulated with 10 ng/ml bFGF for 8 h. Subsequently, the supernatants were collected and VEGF-A was quantified using an ELISA kit (Biosource, Camarillo, CA). Experiments were duplicated and independently repeated three to five times each.

REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Extraction of RNA form and conversion to cDNA was performed. HUVECs in logarithmic growth phase were put into 6-well flat-bottomed tissue culture plates at the density of 2×10^5/ml. For groups of histamine alone, HUVECs were exposed to various concentrations of histamine (0, 0.1, 1, 10, 100 μM) for 24 h. For groups of histamine plus bFGF, HUVECs were initially pretreated with different concentrations of histamine for 24 h and then incubated with 10 ng/ml bFGF for different intervals. Total RNA (0.5 μg) was isolated with Trizol reagent (Invitrogen, Carisbad, CA). Reverse transcription was performed using oligo $(dT)_{18}$ primer and M-MLV reverse transcriptase (Invitrogen) at 37°C for 50 min. The primer sequences were chosen as follows: VEGF primers (sense) 5-GGA GGG CAG AAT CAT CAC GAA G-3 and (antisense) 5-CAC ACA GGA TGG CTT GAA GAT G-3; FGF receptor-1 primers (sense) 5-AAC CCC AGC CAC AAC CCA-3 and (antisense) 5-AAG CTG GGC TGG GTG TCG-3; FGF receptor-2 primers (sense) 5-TCC TAT GAC ATT AAC CGT-3 and (antisense) 5-TTT AAC ACT GCC GTT TAT-3; FGF receptor-3 primers (sense) 5-TTC GAC ACC TGC AAG CCG-3 and (antisense) 5-AG C AGG TCG TGG GCA AAC-3; FGF receptor-4 primers (sense) 5-GCG GCG TCC ACC ACA TTG-3 and (antisense) 5-GTC TGC ACC CCA GAC CC-3; Flt-1 primers (sense) 5-TGA GTG ATG TTG AGG AAG AGG-3 and (antisense) 5-CCA GGT CCC GAT GAA TGC-3; Flk-1/KDR primers (sense) 5-CTG TAT GGA GGA GGA GGA AG-3 and (antisense) 5-CCG TCT GGT TGT CAT CTG CG-3. VEGF, FGF receptors, and VEGF receptors transcripts were amplified on the following thermocycling programs. VEGF: 35 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min; FGF receptors: 40 cycles at 95°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min; VEGF receptors: 40 cycles at 94°C for 1 min, at 57°C for 1 min, and at 70°C for 1 min. The amplified products were electrophoresed with 1% agarose gel and visualized by GoldView™ (SBS Genetech, Beijing, China) and UV irradiation [Bello et al., 2008].

WESTERN BLOT ANALYSIS

Proteins ERK1/2, p38, JNK, IκBα, eNOS, and relative phosphorylated proteins were detected by western blot analysis as described previously [Holden et al., 2007]. GAPDH was used as an internal standard. For groups of histamine alone, HUVECs were exposed to various concentrations of histamine (0, 0.1, 1, 10, 100 μM) for 24 h. For groups of histamine plus bFGF, HUVECs were initially pretreated with different concentrations of histamine for 24 h and then incubated with 10 ng/ml bFGF for different intervals. HUVECs were washed twice by ice-cold PBS buffer (pH 7.2), and proteins were extracted with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.02% NaN_3, 1% NP_{40}) for 30 min on ice. Lysates were clarified by centrifugation at 12,000 rpm for 15 min at 4°C. The concentration of protein in the supernatants was determined by bioinchoninic acid protein (BCA) assay. Then equal amounts of lysate protein were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto PVDF membranes. After blocked with 10% nonfat dry milk for 1 h at room temperature, the membranes were washed three times with PBST buffer and incubated with multiclonal antibodies or phosphor-specific antibodies overnight at 4°C. Subsequently, they were washed three times with PBST buffer and incubated with peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies for 1 h at room temperature. The bands were visualized using film exposure with enhanced chemiluminescence detection reagents.

STATISTIC ANALYSIS

Statistical comparison was carried out with three or more groups using one-way analysis of variance (ANOVA) and Dunnett's test. The data represented mean ± SD of three independent experiments. The values of $P < 0.05$ were statistically significant.

RESULTS

EFFECTS OF HISTAMINE ON BFGF-INDUCED ANGIOGENESIS

Angiogenesis was assayed by a series of experiments, including in vitro proliferation, tube formation of endothelial cells and sprouting

of rat aortic ring. The effects of histamine itself and its combination with bFGF were observed, respectively.

The proliferation of HUVECs was evaluated by MTT assay. Histamine (0.1, 1, 10 μM), incubated with HUVECs for 24 h, did not affect the cell growth. At the concentration of 100 μM, it exhibited inhibitory effect, which might be due to the cytotoxicity (Fig. 1A). However, bFGF (10 ng/ml), incubated with HUVECs for 24 h, could significantly promote the proliferation. Of note, HUVECs, pre-incubated with histamine (0.1, 1, 10 μM) for 24 h, exhibited evident susceptivity to bFGF stimulation. The proliferation indexes in histamine plus bFGF groups were increased by 1.3-, 1.5- and 1.6-folds, respectively, as compared with 1.27-fold of bFGF alone group (Fig. 1B).

The tube formation of HUVECs was tested on the surface of matrigel, which could offer the cells a stroma-like microenvironment. As shown in Figure 1C, cells in control group were scattered on the surface of the gel and had rarely aligned with each other. In histamine (0.1, 1, 10 μM) groups, no striking branching morphogenesis was observed under microscope. bFGF (10 ng/ml) alone treatment for 12 h resulted in a tendency of tube formation, and the movement and reorganization of cell layer could be observed. HUVECs, pre-incubated with histamine (0.1, 1, 10 μM) for 24 h and then stimulated with bFGF (10 ng/ml) for 12 h, showed more evident tube formation as compared with bFGF alone, and robust tube-like structures could be well observed at the group pretreated with 10 μM of histamine (Fig. 1C).

The microvessel sprouting from vascular tissues was tested using rat aortic ring assay. The sprouting process well mimiced several steps of angiogenesis, such as proliferation, migration and tube formation of endothelial cell. Histamine (0.1, 1, 10 μM) alone treatment for 4 days showed little effect on the number of microvessels. Nevertheless, bFGF (10 ng/ml) treatment resulted in developed endothelial sprouts from the aortic ring segments. The

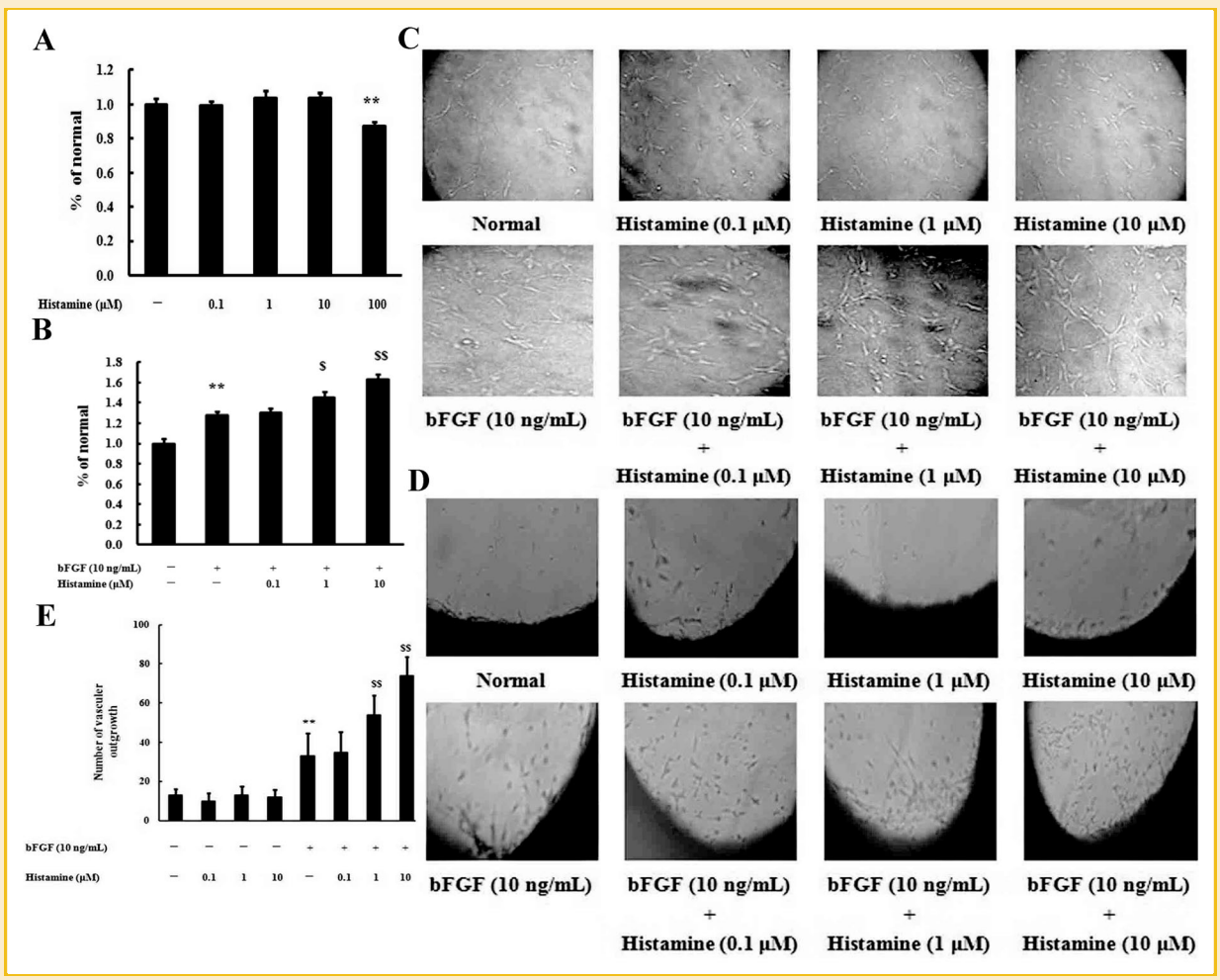

Fig. 1. Effects of histamine on bFGF-induced angiogenesis. A: Effect of histamine on the proliferation of HUVECs. Cells were treated with histamine for 24 h. B: Effect of histamine on the proliferation of HUVECs induced by bFGF. Cells were treated with histamine plus bFGF for 24 h. C: Effect of histamine alone and its combination with bFGF on the tube formation of HUVECs (100×). Cells were treated with histamine for 24 h, and then incubated with or without bFGF for another 12 h. (D and E) Effect of histamine alone and its combination with bFGF on vitro aortic ring sprout. D: Photomicrographs of vascular outgrowth (100×). E: Quantification of the vascular outgrowth. For groups of histamine alone, aorta rings were exposed to various concentrations of histamine (0, 0.1, 1, 10, 100 μM) for 4 days; for groups of histamine plus bFGF, aorta rings were initially pretreated with different concentrations of histamine for 24 h and then incubated with 10 ng/ml bFGF for 3 days. The pictures of microvascular outgrowth were photographed on day 4. Data were expressed as mean ± SD of three experiments. $^{**}P < 0.01$ versus normal; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus bFGF alone.

sprouts could be observed from day 3, and peaked at day 4–6. Notably, aortic rings, pretreated with histamine (0.1, 1, 10 μM) for 24 h and then stimulated with bFGF (10 ng/ml) for 3 days, showed more sprouts as compared with bFGF alone (Fig. 1D,E).

These findings indicated that histamine itself was absent of direct pro-angiogenesis ability. But, it concentration-dependently promoted the responsiveness of endothelial cells to bFGF, and exhibited substantial synergistic effect with bFGF on angiogenesis processes.

EFFECTS OF HISTAMINE ON BFGF-INDUCED VEGF-A EXPRESSIONS IN HUVECS

In addition to direct action via relative receptors, several growth factors and cytokines may exert pro-angiogenesis properties through enhancing the production of VEGF-A, one of the most potent inducer of angiogenesis that participates in and influences the main steps of new vessel development [Nissen et al., 1998; Arai et al., 2006]. The crucial and consequential positions of VEGF support the viewpoint that the amount of VEGF is a kind of reliable index of angiogenesis level. In order to test the possibility that histamine synergized the effects of bFGF through VEGF production, the levels of VEGF-A mRNA and protein in HUVECs were detected by RT-PCR and ELISA, respectively.

As shown in Figure 2A, bFGF (10 ng/ml) stimulation indeed caused a time-dependent VEGF-A mRNA expression in HUVECs. The VEGF-A levels during 2–3.5 h after stimulation were significantly higher compared with untreated cells, whose level peaked at 3 h and increased by around 2.7-fold. Histamine (0.1, 1, 10 μM) itself treatment for 24 h did not affect VEGF-A mRNA expression (data not shown). However, the pretreatment of histamine for 24 h substantially enlarged the VEGF-A expression stimulated by bFGF for 3 h. At concentrations of 1 and 10 μM, histamine enhanced the expressions of VEGF at mRNA and protein levels by approximate 20% and 70%, respectively (Fig. 2B,C). These data demonstrated that histamine was able to synergistically promote bFGF-induced VEGF production in HUVECs.

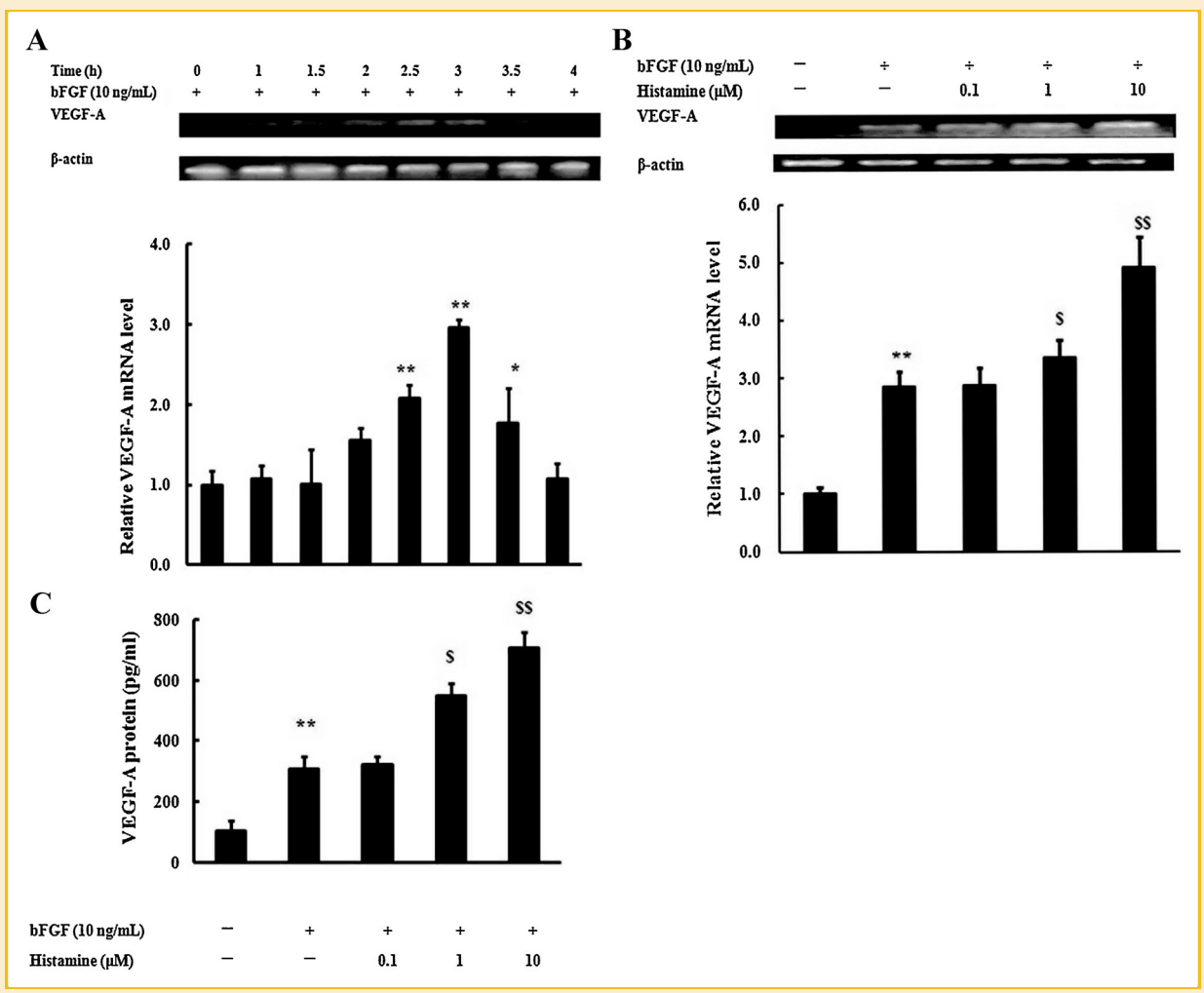

Fig. 2. Effects of histamine on bFGF-induced VEGF-A expressions in HUVECs. A: Time course of bFGF-induced VEGF mRNA expression. B: Effect of histamine on bFGF-induced VEGF mRNA expression. HUVECs were pretreated with histamine for 24 h before stimulation with bFGF for 3 h C: Effect of histamine on bFGF-induced VEGF protein expression. HUVECs were pretreated with histamine for 24 h before stimulation with bFGF for 8 h. The cell supernatants were collected and analyzed by ELISA kits. Data were expressed as mean ± SD of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus bFGF alone.

EFFECTS OF RECEPTOR INHIBITORS ON HISTAMINE-INDUCED VEGF-A EXPRESSIONS IN HUVECS

Histamine exerts biologic function through relevant receptors, including H1–H4 four receptor subtypes. In endothelial cells, H1, and H2 are the predominant histamine receptors. To understand which receptor mediates the synergistic effects of histamine on VEGF-A expression, HUVECs were pretreated with either diphenhydramine (H1 specific blocker) or cimetidine (H2 specific blocker) for 1 h before histamine was added.

As shown in Figure 3, histamine (10 μM) synergistically promoted bFGF-induced VEGF-A mRNA expression by 3.2-fold. Diphenhydramine (30 μM) pretreatment nearly completely reversed the action of histamine, whereas cimetidine (30 μM) pretreatment showed little effect. The findings suggested that H1- but not H2-receptor mediated the synergistic effects of histamine on VEGF-A production caused by bFGF in endothelial cells.

EFFECTS OF HISTAMINE ON BFGF-INDUCED ACTIVATION OF MAPKS AND NF-κB IN HUVECS

In endothelial cells, there are four types of bFGF receptors, that is FGFR (1–4). BFGF interacts with these receptors, and subsequently leads to the activation of multiple signal proteins and transcriptional factors. To recognize the mechanisms underlying the synergistic effects of histamine on bFGF-induced VEGF-A overexpression, impacts on the receptor expressions and the activation of signal pathways were addressed. At first, the effects of histamine on bFGF receptor expressions in HUVECs were examined. The results showed that histamine (10 μM) stimulation for 24 h did not affect mRNA expressions of the four-bFGF receptor subtypes cited above (data not shown).

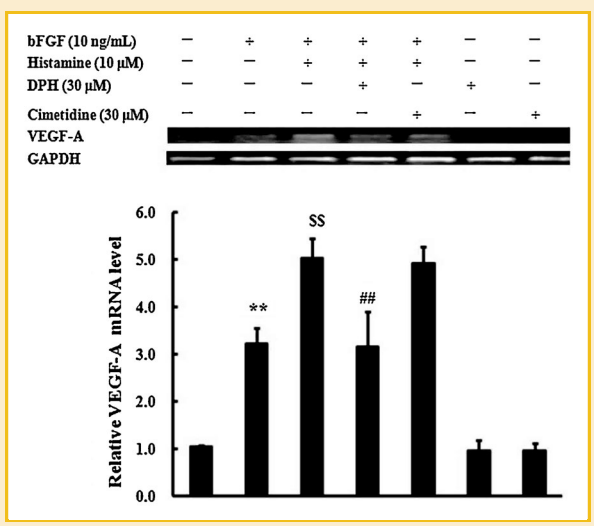

Fig. 3. Effects of receptor inhibitors on histamine-induced overexpression of VEGF-A in HUVECs. Cells were pretreated with diphenhydramine (DPH) or cimetidine for 1 h, followed by histamine for 24 h, and then stimulated with bFGF for 3 h. Data were expressed as mean ± SD of three experiments. $^{**}P < 0.01$ versus normal; $^{\$\$}P < 0.01$ versus bFGF alone; $^{\#\#}P < 0.01$ versus bFGF plus histamine (10 μM).

MAPK pathway, mainly including three kinds of kinases: ERK1/2, JNK, and p38, is well known to regulate bFGF-induced activation of endothelial cells. In this study, bFGF stimulation resulted in blazing phosphorylations of p38, JNK, and ERK in HUVECs, which occurred as early as 5 min, peaked at 10 min (2.6-, 2.9-, and 2.8-folds, respectively) and gradually declined to the base line at 30 min (Fig. 4A). Of note, pretreatment with histamine selectively increased bFGF-induced phosphorylation of p38 rather than ERK1/2 and JNK at 10 min (Fig. 4B), suggesting that p38 pathway might play a crucial role in the synergistic action of histamine on bFGF-induced over-expression of VEGF-A in HUVECs.

NF-κB is also a key regulator for the activation of endothelial cells. As Figure 4C showed, bFGF stimulation led to evident phosphorylation of IκBα, a member of NF-κB family. The phosphorylation peaked at 20 min, and could be remarkably enhanced by histamine pretreatment (1, 10 μM) (Fig. 4D).

SB203580, a selective inhibitor of p38, was used to further identify the participation and importance of p38 in the synergistic action of histamine on bFGF-induced production of VEGF-A. Of interest, SB203580 (5, 10, 20 μM) pretreatment concentration-dependently inhibited the synergistic action of histamine. At 20 μM, SB203580 nearly completely reversed the synergic effect of histamine. Cyclocheximide, a widely used protein synthetic inhibitor, also completely reversed the synergic effect of histamine (Fig. 4E).

Considering that bFGF induced transient activation of p38 and relatively later phosphorylation of IκBα as well as the effect of cyclocheximide, the synergistic mechanisms of histamine against VEGF-A production could be postulated as follows: p38-NF-κB-VEGF-A protein synthesis.

EFFECTS OF HISTAMINE ON BFGF-INDUCED ACTIVATION OF NOS AND COX IN HUVECS

There are reports suggesting the involvements of NOS and COX pathways in the down stream signal transduction of bFGF receptors and subsequent activation of endothelial cells as well as angiogenesis [Muñoz-Chápuli et al., 2004; Yue et al., 2006; Qian et al., 2008; Tsai et al., 2010]. Whether NOS and COX participated in the synergistic effect of histamine on bFGF-induced production of VEGF-A was therefore examined. Figure 5A shows that L-NAME (30 μM), a selective inhibitor of NOS, completely diminished the synergic effect of histamine on VEGF-A expression in HUVECs. In contrast, indomethacin (30 μM), a selective inhibitor of COX, did not exhibit any effect. The findings demonstrated that p38 and NOS/NO rather than COX/prostaglandin E2 (PGE2) pathway were essential for the synergic effect of histamine on VEGF-A expression.

In vascular endothelial cells, eNOS is the key synthase responsible for the production of NO, which plays an important role in both the state of blood vessel vasodilatation and angiogenesis [Li et al., 2003; Yue et al., 2006]. In order to further recognize the participation of NO in the synergic effect of histamine on VEGF-A epression, the direct action of histamine on eNOS was investigated. As Figure 5B shown, histamine (10 μM) substantially increased the expression of eNOS in a time-dependent manner, consistent with previous report by Li et al. [2003], but it did not affect the phosphorylation of eNOS in HUVECs (data not shown). Interestingly,

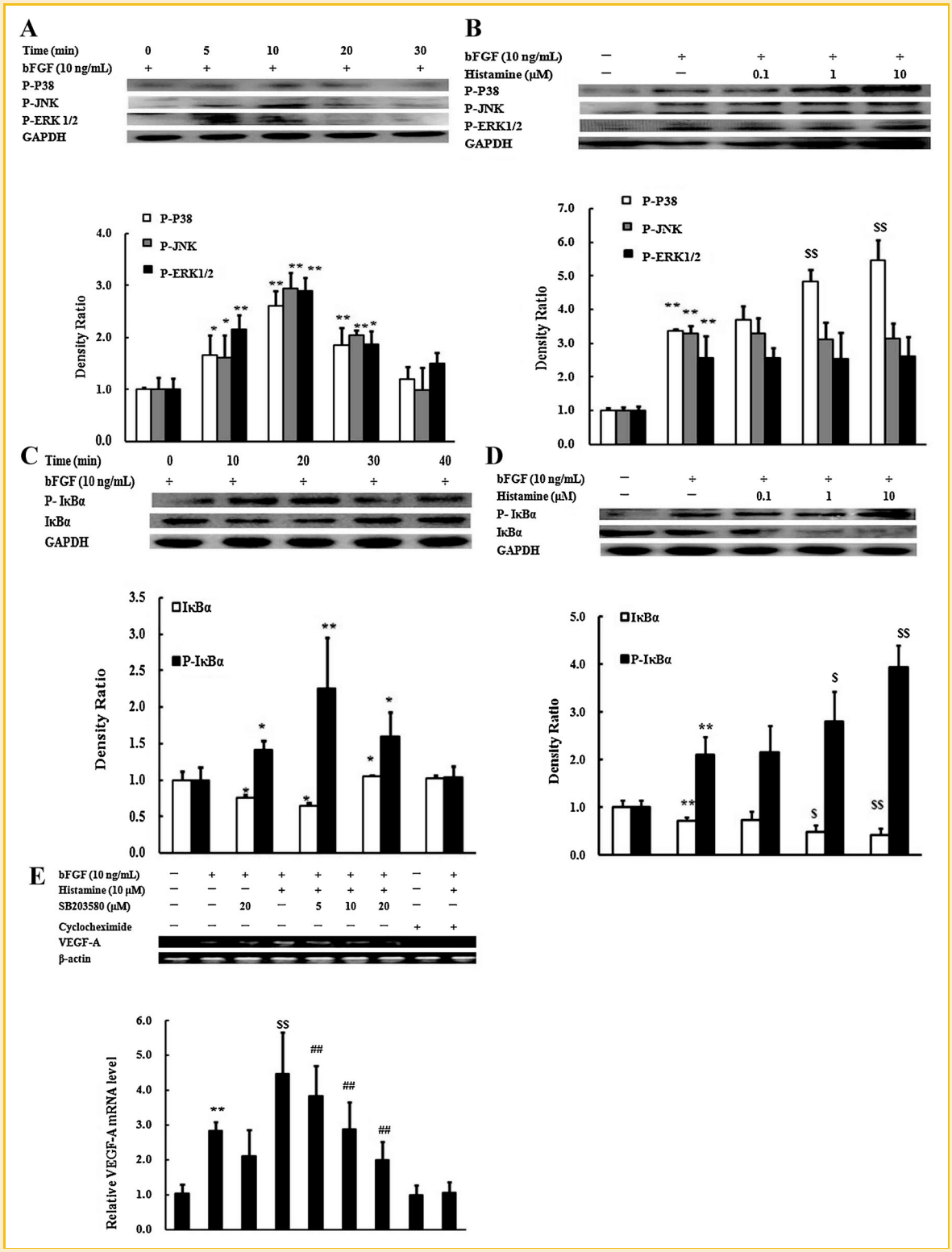

Fig. 4. Effects of histamine on bFGF-induced activation of MAPKs and NF-κB in HUVECs. A: Time course of bFGF-induced MAPK activation. B: Effect of histamine on bFGF-induced MAPK activation. C: Time course of bFGF-induced IκBα activation. D: Effect of histamine on bFGF-induced IκBα activation. E: Effects of SB203580 and cyclocheximide on VEGF mRNA expression induced by histamine plus bFGF. For A, B, C, and D, HUVECs were pretreated with histamine for 24 h, and then stimulated with bFGF at different intervals. For E, HUVECs were pretreated with inhibitors for 1 h, followed by histamine for 24 h, and then stimulated with bFGF for 3 h. Data were expressed as mean ± SD of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus bFGF alone; $^{\#\#}P < 0.01$ versus bFGF plus histamine (10 μM).

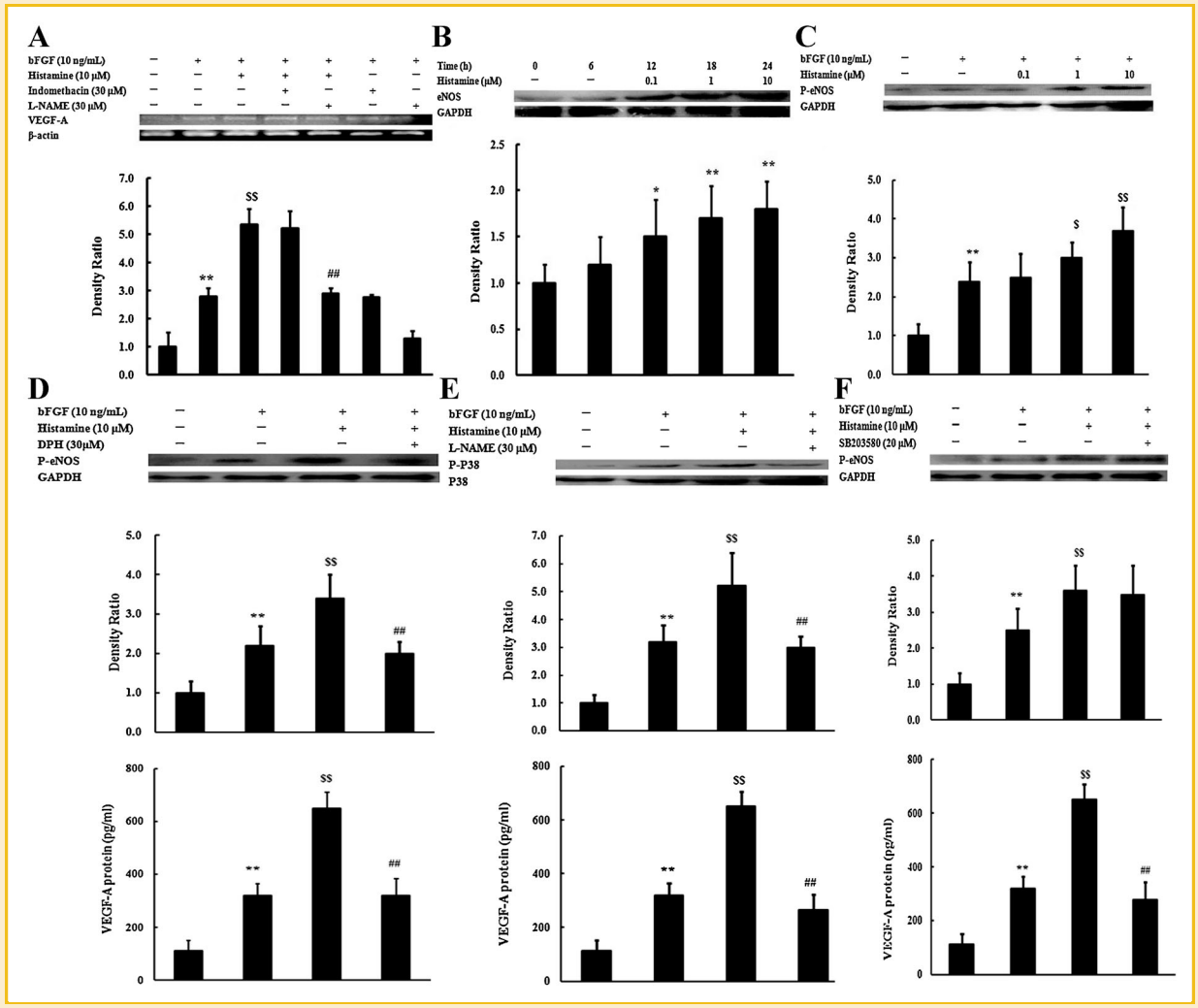

Fig. 5. Effects of histamine on bFGF-induced activation of NOS and COX in HUVECs. A: Effects of L-NAME and indomethacin on VEGF mRNA expression induced by histamine plus bFGF. B: Effects of histamine on eNOS protein expression. C: Effects of histamine on bFGF-induced phosphorylation of eNOS. D: Effects of DPH on the phosphorylation of eNOS and VEGF-A release induced by histamine plus bFGF. E: Effects of L-NAME on p38 activation and VEGF-A release induced by histamine plus bFGF. F: Effects of SB203580 on the phosphorylation of eNOS and VEGF-A release induced by histamine plus bFGF. For A, D, E, and F, HUVECs were pretreated with histamine for 24 h, and then stimulated with bFGF for different intervals as described. Various inhibitors were added 1 h before stimulation. For B, cells were treated with histamine for 24 h. For C, cells were pretreated with histamine for 24 h, and then stimulated with bFGF for different intervals. Data were expressed as mean ± SD of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ versus bFGF alone; $^{\#\#}P < 0.01$ versus bFGF plus histamine (10 μM).

histamine (0.1, 1, 10 μM) could augment bFGF-induced eNOS phosphorylation in HUVECs, and the increasing percentage at 10 μM reached at 80% as compared with bFGF group (Fig. 5C). Moreover, the increased eNOS phosphorylation and VEGF expression by histamine could be abrogated by H1-receptor antagonist diphenhydramine (30 μM) (Fig. 5D).

As shown in Figure 5E,F, either L-NAME (30 μM) or SB203580 (20 μM) could decrease the synergistic action of histamine on bFGF-induced VEGF epression. This meant both eNOS and p38 pathways participated in the synergistic action on VEGF expression, which was also consistent with the conclusion of the result mentioned above. In addition, L-NAME (30 μM) was shown to completely diminish the synergic effect of histamine on bFGF-induced p38 phosphorylation (Fig. 5E), whereas SB203580 (20 μM) failed to affect on the synergic effect of histamine on bFGF-induced eNOS phosphorylation (Fig. 5F). These findings indicated that eNOS located upper stream of p38 in the synergistic effect of histamine on bFGF-induced VEGF expression.

EFFECTS OF HISTAMINE ON BFGF-INDUCED PHOPHORYLATIONS OF VEGF RECEPTORS IN HUVECS

VEGF, secreted from HUVECs, should in turn act on HUVECs by binding with specific receptors VEGFR1 and VEGFR2, and results in the activation of endothelial cells and consequent angiogenesis. In this study, the effects of histamine on the phophorylations of VEGFR1 and VEGFR2 were assessed as well. The results showed that pretreatment with histamine (1, 10 μM) for 24 h significantly enhanced bFGF-induced phosphorylations of both VEGFR1 and VEGFR2 in HUVECs (see Supplementary Materials).

DISCUSSION

Histamine is a major mediator in mast cells, and will be released into the extracellular milieu upon degranulation. It exerts a wide range of bioactivities on several classic physiological and pathological processes, such as inflammation, gastric acid secretion and neurotransmission [Thurmond et al., 2008; Kwon et al., 2011]. During participation in these processes, histamine acts on different types of cells, such as monocytes, macrophages, eosinophils, T hymphocytes, neutrophils, and endothelial cells, to modulate their functions [Li et al., 2001; Thurmond et al., 2008; Kwon et al., 2011]. The best two characterized examples are as follows: (1) Histamine directly acts on vascular smooth muscles cells and endothelial cells leading to vasodilation and more vascular permeability during inflammation. (2) Histamine acts on parietal cells in gastrointestinal system leading to the secretion of H^+ [Thurmond et al., 2008]. Furthermore, it is well known that the diverse bioactivities of histamine are mediated via different receptors, which have four isoforms H1-, H2-, H3-, and H4-receptors. All the isoforms belong to G-protein-coupled receptor (GPCR) family, which means that they not only share conserved motifs common to other monoamine GPCRs, but also are classified by coupling and activating specific G-proteins and then lead to different functions. In these isoforms, H1- and H2-receptors are expressed on many cell types, such as endothelial cells. H3 receptor acts as a neurotransmitter and H4-receptor exists in peripheral blood leukocytes [Nissen et al., 1998; Thurmond et al., 2008].

Recently, histamine has been postulated to be involved in other physiological conditions such as angiogenesis, a growth and remodeling process by which an initial vascular system is modified to form a complex branching network. The process of angiogenesis can be roughly divided into following steps: First, the release of extracellular matrix (ECM) proteases leads to the localized degradation. Then, the local endothelial cells change shape, invade stroma, proliferate, and finally form tubular structures under the control of a huge number of pro- and anti-angiogenic factors which function directly and synergistically [Shahrara et al., 2002; Crivellato et al., 2008]. A direct association between histamine and angiogenesis is the increased level of histamine in the plasma of patients with some solid tumors and the synovial fluid of patients with rheumatoid arthritis, which have similar features such as angiogenesis during their development [Frewin et al., 1986; Thurmond et al., 2008]. Meanwhile, when acting on the key effector in angiogenesis named endothelial cells, histamine induces the release of some proinflammatory factors such as IL-6 and IL-8, and the expressions of several proteins such as TLR and eNOS [Li et al., 2001, 2003; Talreja et al., 2004; Tan et al., 2007].

In order to recognize how histamine works on endothelial cells in more detail, we first examined the single effect of histamine by a series of classic angiogenesis experiments in vitro, including proliferation and tube formation of endothelial cells as well as aortic ring sprouting. The results indicated that histamine itself was absent of direct effect on these angiogenesis steps. Considering the cooperative and synergic action features of many pro-angiogenic factors, we inferred that histamine might work on endothelial cells in cooperation with other pro-angiogenic factors.

VEGF and bFGF are well-identified pro-angiogenic growth factors. Of which, bFGF has been pointed out its original impetus on angiogenesis by binding to relative receptors FGFR (1–4) [Yue et al., 2006], while VEGF plays a secondary role but maintains the angiogenic stage by binding to its receptors VEGFR (flt-1, flk-1/KDR) [Yue et al., 2006]. In the present study, histamine pretreatment did not affect the mRNA expressions of relative receptors during 24 h (data not shown), but it concentration-dependently promoted bFGF-induced proliferation, tube formation and microvasular outgrowth of endothelial cells. This highlighted that histamine was indeed able to enhance the susceptibility of endothelial cells to bFGF and consequent angiogenesis, and bFGF receptors might offer less dedication in the synergetic action of histamine to bFGF.

Besides direct pro-angiogenic action, bFGF may exert action through promoting the production of VEGF, one of the most powerful growth factors in blood vessel development [Nugent and Iozzo, 2000; Yoshiji et al., 2002]. To get insight into the mechanisms of histamine with bFGF, its effect on VEGF expression was investigated. Data of our study showed that histamine could indeed promote bFGF-induced VEGF expression in HUVECs at both gene and protein levels. Furthermore, diphenhydramine (H1-receptor specific antagonist) and cimetidine (H2-receptor specific antagonist) were used to ascertain the participation of histamine receptors. Diphenhydramine (30 μM) nearly completely reversed the synergetic action of histamine upon bFGF-induced VEGF mRNA expression, but cimetidine (30 μM) did not show significant effect. In consistent with this, De Luisi et al. [2009] reported the anti-angiogenic activity of ebastine, a well-known selective second-generation histamine H1-receptor antagonist, and suggested its potential use as an anti-angiogenic molecule besides antihistamine activity for the treatment of allergic diseases. These findings suggested that histamine promoted the pro-angiogenic action of bFGF through synergistically inducing VEGF expression in endothelial cells via H1-receptor, and antihistamines might find their new therapeutic uses in angiogenesis relevent diseases.

As histamine did not affect the expression of bFGF receptors, its synergism on bFGF may results from the crosstalk between signaling molecules at the downstream of histamine receptors and those of FGF receptors. To address this issue, the effects of histamine on bFGF-induced intracellular signal transduction pathways in endothelial cells were investigated. During bFGF-induced activation of endothelial cells and resultant VEGF expression, MAPK, and NF-κB signal pathways are believed to play key roles [Tong et al., 2006; Tabruyn and Griffioen, 2007]. Members of the MAP kinase family, namely, ERK1/2, JNK 1, and p38, are central elements of post-receptor signal transduction pathways in mammalian cells including endothelial cells [Gardner and Johnson, 1996]. Moreover, the activation of NF-κB in endothelial cells was recently reported to aid to the expression of some pro-angiogenic factors such as VEGF and then induce angiogenesis [Flati et al., 2006]. It is well known that NF-κB is one of the most ubiquitous transcription factors and it can coordinate the expression of proinflammatory enzymes and cytokines. In cytoplasm, NF-κB exists as an inactive form through association with IκB family members. IκB kinase (IKK) complex, which is always activated by lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) or bFGF, is needed to phosphorylate and

degrade IκBα, and results in translocation of NF-κB into nucleus to mediate a series of gene transcription [Flati et al., 2006].

In the present study, it was shown that bFGF stimulation led to evident phosphorylations of ERK1/2, JNK, and p38 MAP kinase in HUVECs, and histamine pretreatment significantly facilitated the phosphorylation of p38 MAP kinase. Pretreatment of SB203580 (20 μM), a selective p38 blocker could nearly completely reverse the synergistic action of histamine upon bFGF-induced over-expression of VEGF. It was therefore suggested that p38 MAP kinase might play an especially important role in the synergism of histamine with bFGF. Additionally, histamine was shown to moderately facilitate bFGF-induced phosphorylation and degradation of IκB with a relatively longer activation persistent period (40 min) than that of p38 (30 min), suggesting a downstream location of NF-κB after MAPK phosphorylation. To sum up, the synergistic mechanisms of histamine against bFGF-induced VEGF-A production could be postulated as "p38-NF-κB-VEGF-A protein synthesis".

A variety of studies have proven that NOS and COX signal pathways are also involved in the regulation of bFGF-induced angiogenesis [Muñoz-Chápuli et al., 2004; Qian et al., 2008]. NO plays a direct role in the mechanisms of action of bFGF [Tsai et al., 2010]. In endothelial cells, the production of NO is mainly associated with eNOS. Therefore, the endothelial eNOS/NO pathway is associated with angiogenesis [Tsai et al., 2010]. It has been reported that angiogenic factors (such as VEGF and bFGF) can increase the proliferation, migration, tube formation, and angiogenesis of endothelial cells by inducing AKT-dependent eNOS phoshporylation and NO production [Muñoz-Chápuli et al., 2004; Tsai et al., 2010]. On the other hand, COX, a kind of angiogenesis bystander molecules, belongs to a family of enzymes, which can catalyze the oxygenation of arachidonic acid to prostaglandin G2/H2 (PGG2/H2) [Tan et al., 2007]. BFGF can increase VEGF release in an autocrine manner by increasing COX-2-generated PGE2 in microvascular endothelial cells (MVECs), and subsequently stimulate the proliferation of MVECs and angiogenesis [Yue et al., 2006; Qian et al., 2008].

To clarify the roles of eNOS and COX pathways in the synergism of histamine with bFGF against VEGF expression in endothelial cells, relative inhibitors L-NAME and indomethacin were used, respectively. It was shown that L-NAME could nearly completely diminish the synergic effect of histamine with bFGF on VEGF-A release, while indomethacin could not, suggesting that the synergic effect of histamine with bFGF was related to eNOS but not COX. In addition, histamine could promote bFGF-induced eNOS phosphorylation in HUVECs. And the synergic effects of histamine with bFGF on eNOS phosphorylation and VEGF expression could be totally abrogated by diphenhydramine. The findings highlighted the indispensable role of H1-receptor. Furthermore, the relationship between eNOS and p38 pathways was addressed by using relative inhibitors L-NAME and SB203580, respectively. The results further demonstrated both eNOS and p38 pathways were essential for the synergism of histamine with bFGF on VEGF expression. Additionally, L-NAME was shown to block the synergic effect of histamine on bFGF-induced p38 phosphorylation, while SB203580 was shown to have little effect on eNOS phosphorylation. It was therefore suggested that eNOS phosphorylation probably existed in the upper reach of p38 in the synergism of histamine with bFGF on VEGF release.

According to these findings, it can be speculated that there might be two pathways involved in the synergistic effect of histamine. One pathway is that histamine improves bFGF-induced activation of second messenger signaling pathways: Histamine induces the activation of H1-receptor, and thereby improves the phosphorylation of FGF receptor on phospholipase C (PLC). The latter results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG), and causes calcium mobilization from intracellular stores and activation of protein kinase C (PKC) [Keogh et al., 2002; Hynes and Dey, 2010]. Another pathway is that histamine promotes the function of a major FGF effector, adaptor protein FRS2: Histamine induces the phosphoryation of Grb2 and Src, which provides a docking site for FRS2, and thereby activates MAPK pathways. The two pathways mentioned above converge on MAPK pathways [Keogh et al., 2002; Hynes and Dey, 2010]. p38 MAPK can not only directly transport into nucleus and induce VEGF mRNA transcription, but also mediate the transcriptional activity of NFκB by regulating the degradation of IκBα and the connection between p65 and VEGF promoter [Millette et al., 2005; Panet et al., 2006; Pin et al., 2012].

In conclusion, our study highlighted that histamine was able to synergistically augment bFGF-induced angiogenesis, and this action was linked to overproduction of VEGF in endothelial cells through activating H1- rather than H2-receptor. Multiple signal transduction pathways participated in the synergism, and their sequences were probably as follows: eNOS-p38 MAPK-IκBα. Antihistamines (H1 receptor antagonists etc.) may find their therapeutic potentials in angiogenesis-related diseases.

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