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Expression and Regulation of Endothelial Nitric Oxide Synthase by Vascular Endothelial Growth Factor in ECV 304 Cells

Nitric oxide (NO) seems to play a pivotal role in the vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation. This study was designed to investigate the role and intracellular signal pathway of endothelial nitric oxide synthase (eNOS) activation induced by VEGF. ECV 304 cells were treated with $VEGF_{165}$ and then cell proliferation, eNOS protein and mRNA expression levels were analyzed to elucidate the functional role of eNOS in cell proliferation induced by VEGF. After exposure of cells to $VEGF_{165}$, eNOS activity and cell growth were increased by approximately two-fold in the $VEGF_{165}$-treated cells compared to the untreated cells. In addition, VEGF stimulated eNOS expression at both the mRNA and protein levels in a dose-dependent manner. Phosphatidylinositol-3 kinase (PI-3K) inhibitors were used to assess PI-3K involvement in eNOS regulation. LY294002 was found to attenuate VEGF-stimulated eNOS expression. Wortmannin was not as effective as LY294002, but the reduction effect was detectable. Cells activated by VEGF showed increased ERK1/2 levels. Moreover, the VEGF-induced eNOS expression was reduced by the PD98059, MAPK pathway inhibitor. This suggests that eNOS expression might be regulated by PI-3K and the ERK1/2 signaling pathway. In conclusion, $VEGF_{165}$ induces ECV 304 cell proliferation via the NO produced by eNOS. In addition, eNOS may be regulated by the PI-3K or mitogen-activated protein kinase pathway.

Key Words : *Angiogenesis; Nitric-Oxide Synthase*

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INTRODUCTION

The vascular endothelial growth factor (VEGF) is known to be a vascular permeability factor and an endothelial cell growth factor secreted by both the smooth muscle and endothelial cells. VEGF is a secreted endothelial cell mitogen and play a major role in hypoxia-stimulated angiogenesis (1). Vascular endothelial cells have binding sites with a high affinity for VEGF and VEGF itself is thought to play a major role both in preserving vascular endothelial cells and vascular endothelial cell recovery (2). VEGF is regulated by the Flt-1 and Flk-1/KDR receptors, which are part of the tyrosine kinase family (3, 4). Flt-1, which has a higher affinity for VEGF than Flk-1/KDR, is thought to mainly regulate the formation of cellular architecture, while Flk-1/KDR is involved in cell proliferation (5-8).

However, the subsequent mechanism of signal transduction following the VEGF receptor in vascular cells is uncertain. The effects of VEGF in permeability and vascular tension are associated with nitric oxide (NO) formation (9, 10), and numerous studies have reported a correlation between the endothelial proliferating effect of VEGF with NO (11-13). Angiogenesis associated with NO was investigated in mouse arteries in vitro or in vivo vascular injury experiments. In experiments using arteries or arterioles, VEGF protein expression is increased while the NO metabolite concentration initially decreases and then increases thereafter. However, in arteries injured with balloons, addition of VEGF protein or naked VEGF cDNA re-stimulates endothelial proliferation, and recovers the endothelial cell-dependent vascular response (14, 15). Such effects correlate VEGF with endothelial cell growth, and this new vascular formation is thought to be due to the continuous maintenance of NO formation. Also, the addition of NO synthase (NOS) inhibitors in endothelial cell cultures inhibited VEGF-induced cell growth in addition to the reticular formation of newly synthesized vascular tissues (12).

However, the exact mechanism of action of VEGF in NO formation is still unclear. Recently, several reports have demonstrated that VEGF-stimulated NO production reguires activation of the phosphatidylinositol 3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) signal transduction pathways (16, 17).

This study was designed to elucidate whether the changes

in eNOS activity by VEGF are simply due to variations in the eNOS activation rate, or due to the changes in the eNOS protein and mRNA levels. In addition, whether or not PI-3K, a known crucial factor in cell growth, and extracellular regulated kinase 1/2 (ERK1/2), a MAPK family, are associated with the eNOS protein expression by VEGF was examined.

MATERIALS AND METHODS

Materials

The recombinant VEGF protein, [^{3}H] citrulline, [(α-^{32}P] dCTP, [(γ-^{32}P] ATP, and enhanced chemiluminescence (ECL) reagents were purchased from Amersham (Buckinghamshire, U.K.). M199 and the phosphate-buffered saline were obtained from Gibco BRL (Grand Island, NY, U.S.A.). The fetal calf serum was purchased from Hyclone (Logan, UT, U.S.A.). The rabbit polyclonal eNOS antibodies, and the anti-rabbit IgG peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The phospho-ERK antibodies and phospho-p38 antibodies were purchased from New England BioLab (Beverly, MA, U.S.A.). The LY294002, wortmannin, and PD98059 were purchased from Biomol (Plymouth Meeting, PA, U.S.A.) and were dissolved in dimethyl sulfoxide (DMSO) prior to addition to the cell cultures or enzyme assays; the final concentrations in DMSO were 0.1% or less. Controls using DMSO alone were run in parallel all cases.

Cell Culture

ECV 304 was obtained from the American Type Culture Collection. The cells were cultured in M199 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal calf serum. The cells were grown at 37°C, 5% CO_2 in fully humidified air and subcultured twice weekly. The cells were seeded on 12-well plates at 1×10^5 cells/well or 6-well plates at 5×10^5 cells/well. The cells were stimulated for set times ranging from 1 hr to 24 hr in the presence of VEGF with or without the inhibitors. The viability of the ECV 304 cells in the presence of the VEGF was assessed after different days of incubation by the Trypan Blue exclusion method.

Quantitation of Intracellular NO generation

The eNOS enzyme activity was quantified by L-[^{3}H] citrulline formation from L-[^{3}H] arginine. Briefly, the cells in a given well of the 6-well plate were incubated in 800 μL of a buffer containing 25 mM HEPES, pH 7.3, 109 mM NaCl, 5.4 mM KCl, 0.9 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM glucose at 37°C for 1 hr. The eNOS activity was assayed by adding a mixture of unlabeled 10 μM L-arginine, 10 μCi/mL L-[^{3}H] arginine, and VEGF or the vehicle to the culture. Following incubation at 37°C for 10 min, the cells were washed with ice-cold phosphate-buffered saline (PBS), scraped into 2 mL of a solution containing 20 mM sodium acetate, 1 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA, pH 5.5, followed by sonication. An aliquot was withdrawn to determine the total protein concentration and the total amount of cellular ^{3}H incorporation. The remaining sample was applied to the Dowex 50W-X8 400 column to separate L-[^{3}H] citrulline. The flow-through fraction was analyzed by liquid scintillation counting.

Western blotting and MAPK activation

The ECV 304 cells were plated in a 6-well plate and treated with VEGF with various inhibitors. They were then washed with cold-PBS, scraped off and pelleted at $700 \times g$ and at 4°C. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail). The preparation was then cleared by centrifugation and the supernatant saved as a whole-cell lysate. The proteins (50 μg) were separated by 8% reducing SDS-PAGE and electroblotted in 20% methanol, 25 mM Tris, and 192 mM glycine onto a nitrocellulose membrane. The membrane was then blocked with 5% non-fat dry milk in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) and subsequently incubated with the eNOS antibody for 4 hr. Subsequently, the membrane was washed and incubated for 1 hr with secondary antibodies conjugated to HRP. Finally, the membrane was washed and developed using an enhanced ECL system. The activation of ERK and p38 was determined by Western blotting using antibodies specific for phosphorylated, active forms of the corresponding MAPKs. As loading controls, Western blots were also performed using antibody against total ERK in a 1:1,000 dilution.

RT-PCR

The ECV 304 cells (2×10^6 cells) were cultured and harvested. The cells were then washed three times with PBS containing 2% bovine serum albumin (BSA), and the RNA was isolated using a Tri-Reagent kit (Molecular Research Center, Cincinnati, OH, U.S.A.). A modified reverse transcriptase-polymerase chain reaction (RT-PCR) technique was used to analyze eNOS at the mRNA level. Briefly, the total RNA was reverse transcribed into cDNA using the RT-PCR kit. Oligonucleotide primers for the PCR were designed according to the published sequence for human eNOS (sense primer: ATT ATC CAG AGC GAG AGC CT; antisense primer: TCT TGC CAC CAC TGT GTT GT). The PCR conditions for eNOS production was 24 cycles of denaturation (95°C/1 min), annealing (60°C/1 min), and extension (72°C/1 min) in the

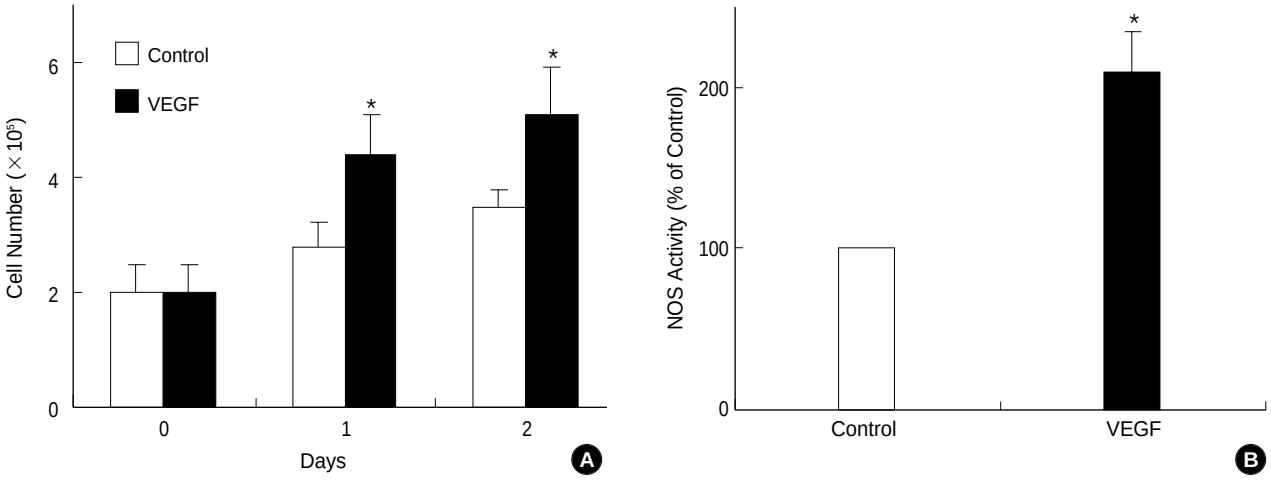

Fig. 1. Effect of VEGF on cell growth and NOS activity. (A) Confluent ECV 304 cells stimulated with the VEGF (20 ng/mL) for the different days. After treatment with VEGF, the cells were collected and counted the viable cells using Trypan Blue exclusion method. (B) ECV 304 cells were cultured for 24 hr at 37℃ in serum free media in the absence or presence of VEGF (20 ng/mL). Afterward, cells were collected and assayed for NOS activity. The results for NOS activities are average values±SD from three independent experiments. *p<0.05, compared with day 0 or control.

presence of 2.5 mg $MgCl_2$, followed by a final extension at 72℃ for 20 min. Oligonucleotide primers for β-actin were used as internal control for RT-PCR. The PCR products were separated by electrophoresis using a 2% agarose gel in TBE buffer containing 50 ng/mL of ethidium bromide.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 for Windows. Data were given as mean±standard deviation and comparison of the two groups was made by unpaired Students t test. A p-value of <0.05 was considered significant.

RESULTS

Initially, the ECV 304 cells were exposed to 20 ng/mL $VEGF_{165}$ to determine the effect of VGEF on cell growth. The $VEGF_{165}$ protein was found to significantly increase the number of cells by almost 2.2 times after 24 hr, and by 2.5 times after 48 hr (Fig. 1A).

To investigate the effect of $VEGF_{165}$ on eNOS activity, after treating 20 ng/mL $VEGF_{165}$ in an arginine substrate for 24 hr, the concentration of citrulline produced by the NOS enzyme was measured. The results showed that NOS enzyme activation was increased by two-fold in the cells treated with the $VEGF_{165}$ protein, compared with the control group that was not treated with the $VEGF_{165}$ protein (Fig. 1B).

To determine whether the increase in NOS activity observed in the ECV 304 group was the result of eNOS protein expression, the expression of the eNOS and inducible NOS (iNOS) protein was measured after exposing the ECV 304 cells to $VEGF_{165}$ concentrations ranging from 5 ng/mL to

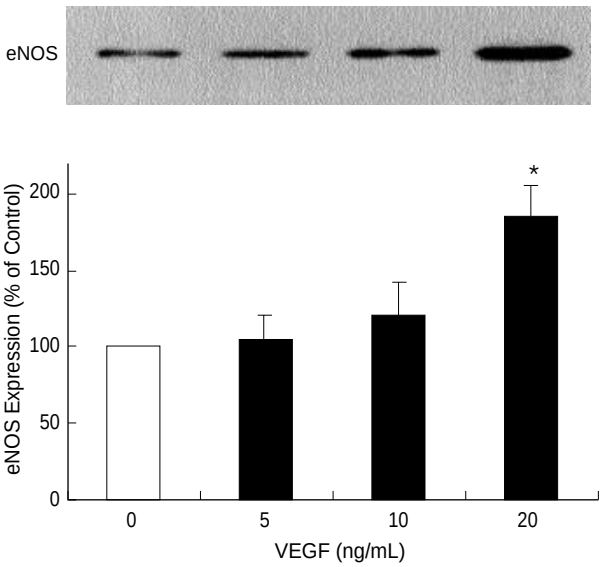

Fig. 2. Effect of VEGF on eNOS expression in ECV 304 cells. Confluent ECV 304 cells were incubated with the indicated concentrations of VEGF for 24 hr. The cells were plated, and the expression of eNOS was determined by Western blot analysis. The eNOS protein data are representative of three independent experiments. The amounts of eNOS protein were quantified using scanning densitometry and expressed relative to the densities of control cells. *p<0.05 compared with control (0 ng/mL).

20 ng/mL for 24 hr. In the cells treated with the $VEGF_{165}$ protein, eNOS protein expression (about 130 kDa) was increased in a dose-dependent manner, and eNOS protein expression in the endothelial cells exposed to 20 ng/mL $VEGF_{165}$ for 24 hr increased by approximately two-fold compared with the control cells (Fig. 2). However, iNOS expression was not

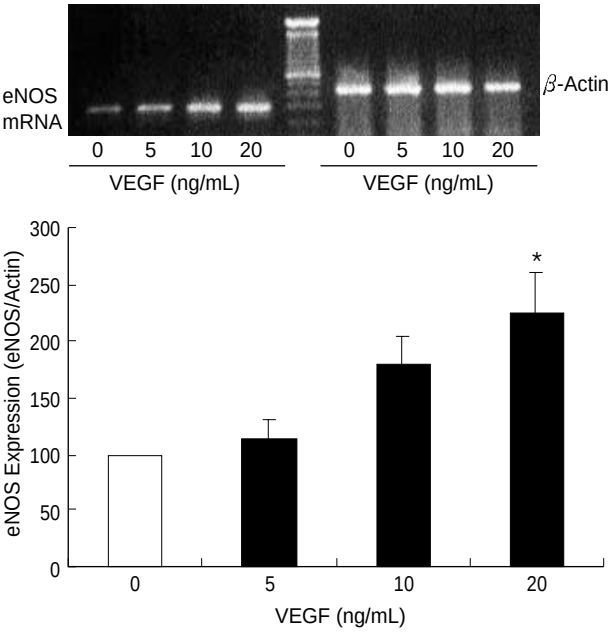

Fig. 3. Effect of VEGF on induction of eNOS mRNA in ECV 304 cells. Confluent ECV 304 cells were treated with various concentrations of VEGF for 24 hr. The mRNA levels of eNOS and β-actin were determined by RT-PCR. The amounts of eNOS mRNA were quantified using scanning densitometry and expressed relative to the densities of β-actin. *p<0.05 compared with control (0 ng/mL).

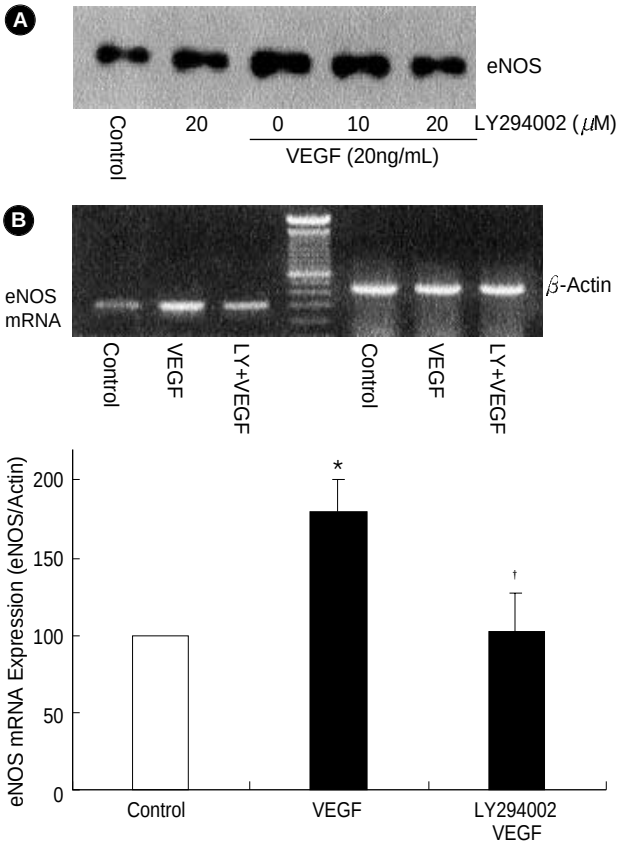

Fig. 4. Effect of LY294002 on VEGF-induced eNOS protein and mRNA expression. Confluent ECV 304 cells were pretreated with LY294002 (25 μM) for 30 min and then stimulated with the indicated concentrations of VEGF for 24 hr. (A) eNOS expression as determined by Western blot analysis. (B) Total RNA was isolated and mRNA was determined by RT-PCR. The amounts of eNOS mRNA was quantified using scanning densitometry and expressed relative to the densities of β-actin. *p<0.05 and †p<0.05 compared with control and VEGF, respectively.

detected.

To examine whether the increased eNOS protein and the enzyme activation was due to the increase in the mRNA, the amount of mRNA was determined by RT-PCR. After separating the total RNA from the cells harvested after $VEGF_{165}$ protein treatment (0-20 ng/mL) for about 6 hr, RT-PCR was conducted using VEGF-specific primers. The result showed that the amount of β-actin expression used as an internal control was constant, while 296 bp bands that were thought to be the product of eNOS PCR increased by 1.1, 1.8, and 2.2 times when exposed to VEGF concentrations of 5, 10, and 20 ng/mL, respectively (Fig. 3).

To examine the involvement of PI-3K in eNOS expression by VEGF, the endothelial cells were exposed to $VEGF_{165}$ after 1 hr pretreatment with LY294002 or wortmannin, which are well known as PI-3K inhibitors. In the cells pretreated with 10 μM or 20 μM of LY294002, the eNOS expression induced by $VEGF_{165}$ was suppressed depending on the concentration of the inhibitor (Fig. 4A). In the group not pretreated with LY294002, eNOS mRNA expression increased by 1.7 times, whereas in the group pretreated with 20 μM of LY294002, it decreased to the same level as of the control group (Fig. 4B). This shows that PI-3K inhibitor inhibits eNOS mRNA expression by $VEGF_{165}$. Furthermore, eNOS protein expression by $VEGF_{165}$ increased by 2.2 times, compared with 1.6 times when it was exposed to $VEGF_{165}$ after pretreatment with wortmannin, the other PI-3K inhibitor (Fig. 5).

Because there are some reports showing that the eNOS activation in endothelial cells by $VEGF_{165}$ is controlled by ERK1/2, which is a downstream molecule and a type of MAPK, the effect of kinase phosphorylation on eNOS expression was examined. eNOS protein expression by $VEGF_{165}$ was measured after pretreatment with PD98059, which is a known ERK1/2 cascade inhibitor. As shown in Fig. 6, PD98059 pretreatment suppressed the VEGF-mediated eNOS expression (Fig. 6).

To examine whether or not eNOS expression by PD98059 correlates with ERK phosphorylation, immunoblotting was carried out using antibodies for phospho-ERK, an activated form of ERK. As a result, ERK phosphorylation was increased by the $VEGF_{165}$. However, there was no significant change in p38 MAPK phosphorylation (Fig. 7). Furthermore, LY 294002 or PD9805 attenuated the ERK phosphorylation

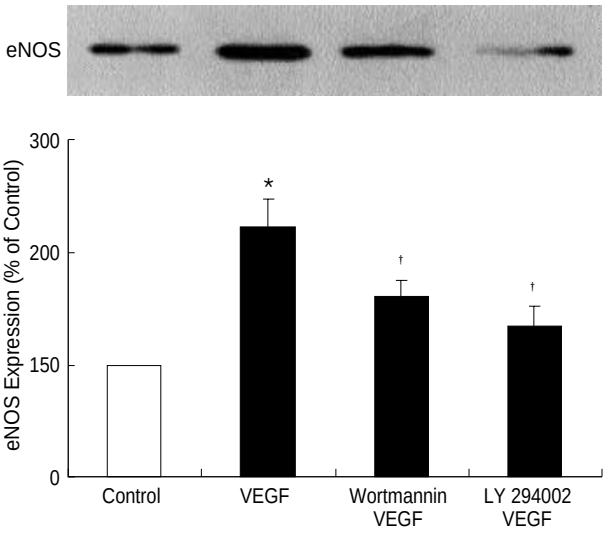

Fig. 5. Effect of the phosphatidylinositol 3 kinase inhibitors on VEGF-induced eNOS expression. Confluent ECV 304 cells were pretreated with LY294002 (25 μM) or wortmannin (100 nM) for 30 min and then stimulated with the VEGF (20 ng/mL) for 24 hr. eNOS expression was determined by Western blot analysis. The eNOS protein data are representative of three independent experiments. $^{*}p<0.05$ and $^{\dagger}p<0.05$ compared with control and VEGF, respectively.

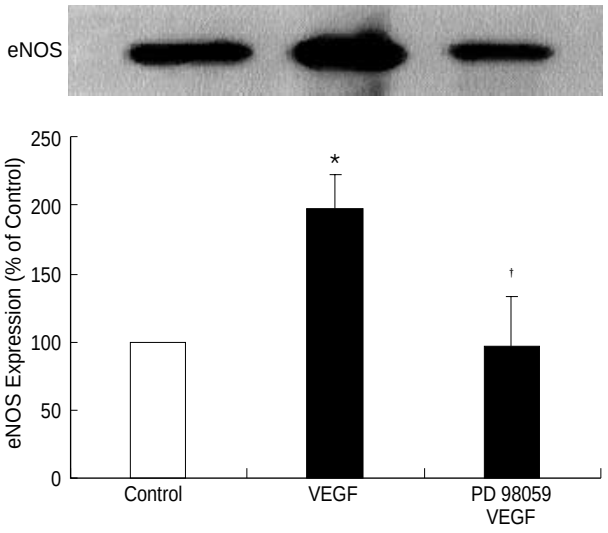

Fig. 6. Effect of PD98059 on the VEGF-induced eNOS expression. Confluent ECV 304 cells were incubated for 30 min in the absence or presence of PD98059 (50 μM) prior to the addition of VEGF (20 ng/mL). eNOS expression was determined by Western blot analysis. The eNOS protein data are representative of three independent experiments. $^{*}p<0.05$ and $^{\dagger}p<0.05$ compared with control and VEGF, respectively.

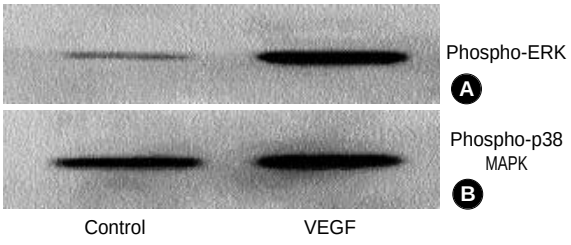

Fig. 7. Effect of VEGF on ERK and p38 MAPK phosphorylation. ECV 304 cells were stimulated with VEGF (20 ng/mL) for 10 min and harvested. Whole cell lysates were resolved by SDS-PAGE followed by Western blot analysis using a set of antibodies that recognize either phosphorylated ERK or phosphorylated p38 MAPK. The data are representative of three independent experiments.

Fig. 8. Effect of inhibitors of VEGF-induced ERK phosphorylation. ECV 304 cells were pretreated with LY294002 (25 μM), wortmannin (100 nM) or PD98059 (50 μM) for 30 min and then stimulated with VEGF for 10 min. Whole cell lysates were resolved by SDS-PAGE and analyzed by Western blot analysis using phospho-ERK antibody. The data are representative of three independent experiments.

by VEGF (Fig. 8).

DISCUSSION

Recent studies have shown that VEGF increases NO production in rabbit or human endothelial cells, and NO is thought to be an important mediator in VEGF-induced endothelial cell proliferation (16, 18). For the basic mechanism of the induction of NO by VEGF, some reports have shown the inhibition of VEGF-induced endothelial cell proliferation by the NOS inhibitor, the inhibition of reticular formation of endothelial cells in three-dimensional collagen gel (12, 19), and that NO produced by substance P or VEGF stimulates bovine endothelial cell proliferation (12, 20). Using ECV 304 endothelial cells, we observed the proliferation of endothelial cells induced by VEGF and an increase in NOS activity. This suggests that NO is an important mediator in VEGF-induced endothelial proliferation.

The fact that the increase of NO through a brief exposure to VEGF is reduced by the tyrosine kinase inhibitor, calcium-chelating compounds, and PI-3K suggests that the NO increase is associated with an activation of tyrosine kinase and PI-3K and an increase in the cellular calcium concentration

(18, 19). Thus, the exposure of human umbilical vein endothelial cells to VEGF results in an increase in the cellular calcium concentration, while treatment with calcium-chelating compounds or calmidazolium results in a decrease of VEGF-induced NO production (9, 19). On the other hand, the chief mechanism of NO production through 24 hr or more of continuous VEGF stimulation is thought to be due to an increase in NOS protein synthesis (7, 19, 21). It was thought that eNOS (type III NOS) is always activated in endothelial cells and its enzymatic activation is partially regulated by intercellular Ca^{2+}, while iNOS (type II NOS) is regulated at the transcription level. However, recent studies have suggested that eNOS protein expression can also be regulated by physical stress or hormone. The increase in eNOS expression by such stimuli as the transforming factor β (22), a basic fibroblast growth factor (23, 24), fluid shear stress (25), and hypoxia (26) has been reported. Moreover, continuous physical exercise on dogs has been shown to increase eNOS expression and the consequent benefits of exercise on the vascular system has been reported (27). This study confirms that the increase of eNOS protein by VEGF at the mRNA level. The results also show that the increase in NO synthesis is through to be not only due to a simple increase in NOS activity, but also due to an increase in the NOS protein at the transcriptional level. iNOS expression was not increased by VEGF in contrasts to Papapetropoulos et al. (19).

Tyrosine kinase is known to participate in the signal transduction and expression of eNOS induced by VEGF. In human endothelial cells, tyrosine kinase inhibitors such as genistein and geldanamycin inhibit NO synthesis through VEGF. At least two receptors, Flt-1 and Flk-1/KDR, are involved in the mechanism of action of VEGF and the cells expressing Flk-1/KDR show more cell structural changes and cell proliferation than those with Flt-1 (5). However, Flk-1/KDR receptor activation through VEGF shows increases in eNOS and iNOS protein expression, but Flt-1 receptor stimulation does not increase NOS protein expression (21). Each one of these receptors has endogenous tyrosine kinase activity. In addition to these receptors, at least 11 proteins, such as phospholipase C-γ, PI-3K, Ras GTPase activating protein (GAP), and Nck (oncogenic adaptor protein), are activated through tyrosine phosphorylation (28). Among the tyrosine phosphorylated proteins, there is also ERK1/2, a number of the MAPK family. In human umbilical vein endothelial cells, VEGF increases tyrosine phosphorylation of the Flk-1/KDR receptors and both receptors phosphorylate and activate the p85, a regulating unit of PI-3K. It then activates MAPK (29). ERK1/2 plays a central role in the growth and proliferation of various cells, in the increase of eNOS expression through VEGF treatment, and consequently in the neovascular formation by VEGF. Studies have shown that the pretreatment with PI-3K inhibitors in human umbilical vein endothelial cells inhibits NO formation through VEGF (19). However, the actual mechanism of PI-3K-associated NO synthesis is still unknown. It is also unclear whether this is due to a simple increase in NOS activity or due to changes in NOS protein synthesis. The present study revealed that the pre-treatment with PI-3K inhibitor, LY294002 or wortmannin, attenuated the eNOS expression in response to VEGF. LY294002 showed a higher degree of inhibition than wortmannin. Furthermore, the pre-treatment with the ERK1/2 inhibitor, PD98059, decreased eNOS expression by VEGF and phosphorylated ERK1/2. Both PI-3K inhibitors, LY 294002 and wortmannin, inhibited ERK1/2 phosphorylation by VEGF. These results suggest that both PI-3K and ERK1/2 pathways play a major role in NOS protein synthesis by VEGF.

In conclusion, NOS protein synthesis is one of the key mechanisms responsible for the increase in NOS activity induced by VEGF and PI-3K and ERK1/2 are deeply involved in VEGF-induced eNOS expression in ECV 304 cells.

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