Thymosin Beta-4, Actin-Sequestering Protein Regulates Vascular Endothelial Growth Factor Expression via Hypoxia-Inducible Nitric Oxide Production in HeLa Cervical Cancer Cells

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Abstract

Vascular endothelial growth factor (VEGF) is an important regulator of neovascularization. Hypoxia inducible nitric oxide (NO) enhanced the expression of VEGF and thymosin beta-4 (Tβ4), actin sequestering protein. Here, we investigated whether NO-mediated VEGF expression could be regulated by Tβ4 expression in HeLa cervical cancer cells. Hypoxia inducible NO production and VEGF expression were reduced by small interference (si) RNA of Tβ4. Hypoxia response element (HRE)-luciferase activity and VEGF expression were increased by the treatment with N-(β-D-Glucopyranosyl)-N2-acetyl-S-nitroso-D, L-penicillaminamide (SNAP-1), to generate NO, which was inhibited by the inhibition of Tβ4 expression with Tβ4-siRNA. In hypoxic condition, HRE-luciferase activity and VEGF expression were inhibited by the treatment with N^G-monomethyl-L-arginine (L-NMMA), an inhibitor to nitric oxide synthase (NOS), which is accompanied with a decrease in Tβ4 expression. VEGF expression inhibited by L-NMMA treatment was restored by the transfection with pCMV-Tβ4 plasmids for Tβ4 overexpression. Taken together, these results suggest that Tβ4 could be a regulator for the expression of VEGF via the maintenance of NOS activity.

Key Words: VEGF, Thymosin beta-4, Nitric oxide, Hypoxia, HIF-1α

INTRODUCTION

Thymosin beta-4 (Tβ4) is a small and naturally occurring 5 kDa peptide with 43 amino acids present in all cells except erythrocytes (Low *et al*., 1981; Huff *et al*., 2001). Tβ4 protein was cross-linked to monomer G-actin (Safer *et al*., 1991). Tβ4 has multiple diverse cellular functions including anti-apoptosis to an external stress (Sosne *et al*., 2004), paclitaxel-resistance through ROS production (Oh *et al*., 2006; Moon *et al*., 2007; Oh *et al*., 2010), and HIF-1α stabilization through Erk activation (Oh *et al*., 2008) via depolymerization of F-actin (Brakebusch and Fassler, 2005; Zvaifler, 2006). In addition, Tβ4 regulates cancer cell migration through various signaling pathways (Moon *et al*., 2010; Im *et al*., 2012; Ryu *et al*., 2012). Tβ4 triggers epithelial-mesenchymal transition (Huang *et al*., 2007), malignant progression and invasion in colon adenocarcinoma (Wang *et al*., 2003; Wang *et al*., 2004).

Vascular endothelial growth factor (VEGF) plays an important role in new blood vessel formation known as angiogenesis. Hypoxia-inducible factor (HIF)-1α stimulates VEGF gene transcription (Richard *et al*., 1999). Cytosolic HIF-1α protein is rapidly degraded by the ubiquitin-proteasome pathway in normoxia (Salceda and Caro, 1997) but its degradation is inhibited in hypoxia (Huang *et al*., 1998). VEGF up-regulation requires Tβ4 expression, which is enhanced in hypoxic condition (Gnecchi *et al*., 2006; Smart *et al*., 2007). Tβ4 also increased HIF-1α protein level and HRE activity in VEGF promoter (Oh *et al*., 2008). Tβ4-associated VEGF expression is indirectly mediated by HIF-1α stability (Jo *et al*., 2010). However, it is not enough information to connect Tβ4 and VEGF expression.

Nitric oxide (NO) is an uncharged free radical and synthesized from the amino acid L-arginine by NO synthase (Marsden *et al*., 1993; Chartrain *et al*., 1994; Hall *et al*., 1994). NO is a mediator of diverse physiological cellular functions including vasodilation, neurotransmission and anti-platelet aggregation (Moncada and Higgs, 2006). NO also plays a role in various cellular effects leading to DNA damage, cell death and anti-apoptosis, which is dependent on cellular NO concentration (Wink *et al*., 1998). In addition, Previous report showed that paclitaxel induces VEGF expression through the production of

reactive oxygen species (ROS), which lead to drug resistance (Kim *et al.*, 2008). NO production is also increased in tumor cells under hypoxia condition (Maulik and Das, 2002), which upregulate VEGF (Xu *et al.*, 2002; Fukumura *et al.*, 2006; Hussain *et al.*, 2008). However, little has been known about whether NO-mediated VEGF expression is controlled by Tβ4 expression under hypoxic condition.

Here, we studied whether VEGF expression in HeLa cervical cancer cells could be regulated by hypoxia-inducible NO via Tβ4 expression. Our data suggest that Tβ4 could be a regulator for the expression of VEGF through NO production.

MATERIALS AND METHODS

Reagents
Anti-rabbit antibodies to HIF-1α or VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies that were reactive with actin and α-tubulin were obtained from Sigma-Aldrich (St. Louis, MO, USA). N^G-monomethyl-L-arginine (L-NMMA) was obtained from Sigma-Aldrich (St. Lois, MO, USA). N-(β-D-Glucopyranosyl)-N_2-acetyl-S-nitroso-D,L-penicillaminamide (SNAP-1) was obtained from Calbiochem (La Jolla, CA, USA). Except where indicated, all other materials including LiCl are obtained from Sigma-Aldrich chemical company (St. Louis, MO, USA).

Cell culture
HeLa cells were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) cell bank (Daejeon, Korea). Cells were maintained and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were incubated under normoxia CO_2 incubator with 5% CO_2 and 95% air at 37°C. Cells were also incubated under hypoxia (0.5% O_2) in an anaerobic incubator (Forma Scientific, Marietta, OH, USA) with 5% CO_2, 10% H_2, and 85% N_2 at 37°C for an appropriate time. Then, cells were incubated at 37°C in an atmosphere of humidified normoxia incubator with 5% CO_2 and 95% air.

Nitrite measurement
To assess NO production, accumulated nitrites were measured in the cell supernatant by the Griess reaction (Moon *et al.*, 2011). In brief, 100 μl of supernatant from each well were mixed with 100 μl of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 2% phosphoric acid) on 96-well microtiter plates. Absorbance was read at 540 nm, using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Hypoxia response element (HRE) reporter assay
HRE reporter plasmid 5x VEGF-HRE-pSV40 min that was generated by cloning five tandem couples of HRE derived from the human VEGF promoter into the BglIII site of pGL3 was kindly provided from Dr. Dong-Soo Im, KRIBB (Taejeon, Korea) (Cho *et al.*, 2004). To measure the activity of VEGF transcription, confluent Hela cells were transfected with VEGF-HRE-pSV40 min plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Reverse transcriptase polymerase chain reaction (RT-PCR)
Total RNA was isolated from HeLa cells using TRIZOL reagent (Invitrogen, Calsbad, CA, USA). cDNA was synthesized from 1 μg of total RNA, using oligo-dT$_{18}$ primers and reverse transcriptase in a final volume of 20 μl (Bioneer, Taejeon, Korea). For standard PCR, one μl of the first strand cDNA product was then used as a template for PCR amplification with Taq DNA polymerase (Bioneer, Taejeon, Korea). PCR amplification proceeded as follows using oligonucleotides specific for human Tβ4 (forward: 5'-atg tct gac aaa ccc gat atg gc-3', reverse: 5'-tta cga ttc gcc tgc ttg ctt c-3'), VEGF (forward: 5'-tga cag gga aga gga gga ga-3', reverse: 5'-tgg ttt caa tgg tgt gag ga-3'), and GAPDH (forward: 5'-gaa ggt gaa ggt cgg agt c-3', reverse: 5'-gaa gat ggt gat ggg att tc-3'). PCR products were detected by running in 1.2% agarose gel electrophoresis.

Western blot analysis
Western blot analysis was carried out according to standard protocol. HeLa cells treated with various experimental condition were harvested and then lysed in ice-cold lysis buffer, containing 0.5% Nonidet P-40 (vol./vol.) in 20 mM Tris-HCl, at a pH of 8.3; 150 mM NaCl; protease inhibitors [2 μg/ml aprotinin, pepstatin; 1 μg/ml leupeptin; 1 mM phenylmethyl sulfonyl fluoride (PMSF)] and 1 mM Na_3VO_4, phospatase inhibitor. Lysates were incubated for 1 h in ice prior to centrifugation at 13,000 rpm for 20 min at 4°C. The protein concentration of the sample was measured using Bio-Rad protein assay dye reagent. Proteins in the supernatant were denatured by boiling for 5 min in Sodium Dodecyl Sulfate (SDS) sample buffer. Sample amount of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes by electro-blotting. Following this transfer, equal loading of protein was verified by Ponceau S staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Tween 20), then incubated with the indicated antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL). Immune-reactive bands were detected using X-ray film.

Statistical analyses
Experimental differences were tested for statistical significance using ANOVA and Students' *t*-test. *p* value of <0.05 was considered to be significant.

RESULTS

Tβ4 regulates NO production and VEGF expression under hypoxic condition
Given that Tβ4 expression and nitric oxide (NO) production were increased under hypoxia condition (Maulik and Das, 2002; Moon *et al.*, 2010), we also examined an increase in Tβ4 expression (Fig. 1A) and NO production (Fig. 1B) in HeLa cervical cancer cells. Hypoxia condition was confirmed by the increase in HIF-1α and VEGF protein level in HeLa cells under hypoxia condition (Fig. 1C). Then, to examine the effect of Tβ4 on VEGF expression, Tβ4-siRNA was used to inhibit Tβ4 expression (Fig. 1D). NO production in HeLa cells under hypoxia condition was inhibited by the transfection with Tβ4-siRNA cells (Fig. 1E). We also observed that HIF-1α and
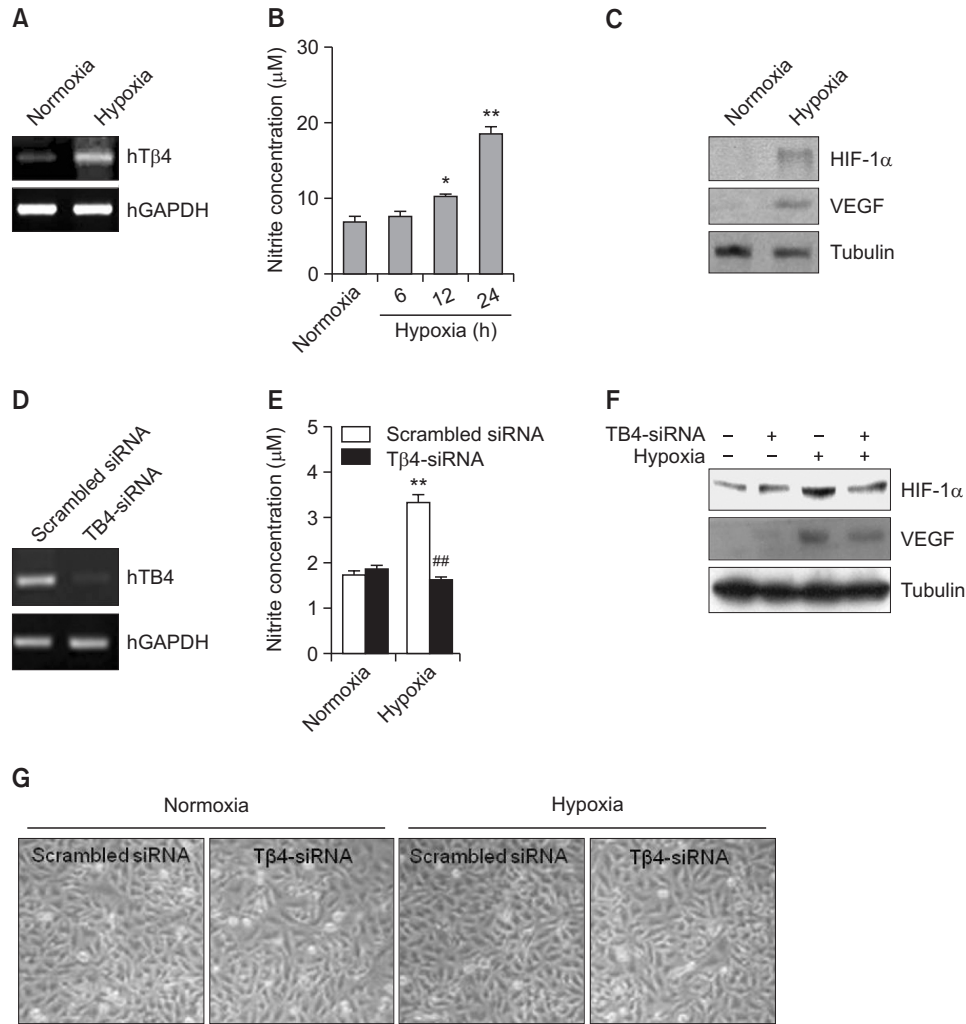
Fig. 1. VEGF is increased by Tβ4 expression under hypoxic condition. (A-C) HeLa cells were incubated under normoxia or hypoxia condition. RNA was purified with TRIZOL reagent. Tβ4 transcript level was measured by RT-PCR (A). NO production was detected as nitrite accumulated in culture supernatant by using Griess reagents. Data in bar graph represent mean ± SED. *$p<0.05$; **$p<0.01$, statistical significance vs. normoxia control group (B). HIF-1α and VEGF in cell lysate were detected by western blot analysis (C). (D-G) Tβ4 expression in HeLa cells was inhibited by the transfection with Tβ4-siRNA. RNA was purified with TRIZOL reagent. Tβ4 transcript level was measured by RT-PCR (D). Cells were incubated under normoxic or hypoxic condition (E-G). NO production was detected as nitrite accumulated in culture supernatant by using Griess reagents. Data in bar graph represent mean ± SED. **$p<0.01$, statistical significance vs. normoxia control group. ##$p<0.01$, statistical significance vs. scrambled siRNA-treated group under hypoxic condition (E). HIF-1α and VEGF protein levels in cell lysates were detected by western blot analysis (F). Cellular morphology was photographed with a phase-contrast microscope. Pictures were taken at the same magnification, 200x. Data are representative of four experiments (G).

VEGF protein level increased under hypoxic condition was attenuated by the inhibition of Tβ4 expression with Tβ4-siRNA cells (Fig. 1F). No changes in cellular morphology were detected in cells transfected with Tβ4-siRNA. However, a little reduction of cell density was observed by the incubation of Tβ4-siRNA-transfected cells under hypoxic condition (Fig. 1G). It suggests that hypoxia-inducible Tβ4 could be involved in VEGF expression.

SNAP-1, NO donor-mediated VEGF expression is dependent on Tβ4 expression

To confirm the effect of NO on VEGF through Tβ4 expression in HeLa cells, we used N-(β-D-Glucopyranosyl)-N$_2$-acetyl-S-nitroso-D,L-penicillaminamide (SNAP-1) as NO donor (Fig.

2A). SNAP-1 treatment enhanced VEGF transcription as judged by hypoxia response element (HRE) reporter activity in VEGF promoter (Fig. 2B). NO-mediated VEGF expression increased by SNAP-1 was attenuated by the transfection with Tβ4-siRNA (Fig. 2C, D). Inhibition of Tβ4 expression was proved by examination (Fig. 2D). Data demonstrate that NO-mediated VEGF expression is dependent of Tβ4 expression

Hypoxia-induced VEGF expression is inhibited by L-NMMA, NOS inhibitor

To test the effect of hypoxia-inducible NO on VEGF expression through Tβ4 expression in HeLa cells, we treated cells with N^G-monomethyl-L-arginine (L-NMMA). Hypoxia response element (HRE) reporter activity in VEGF promoter was re-
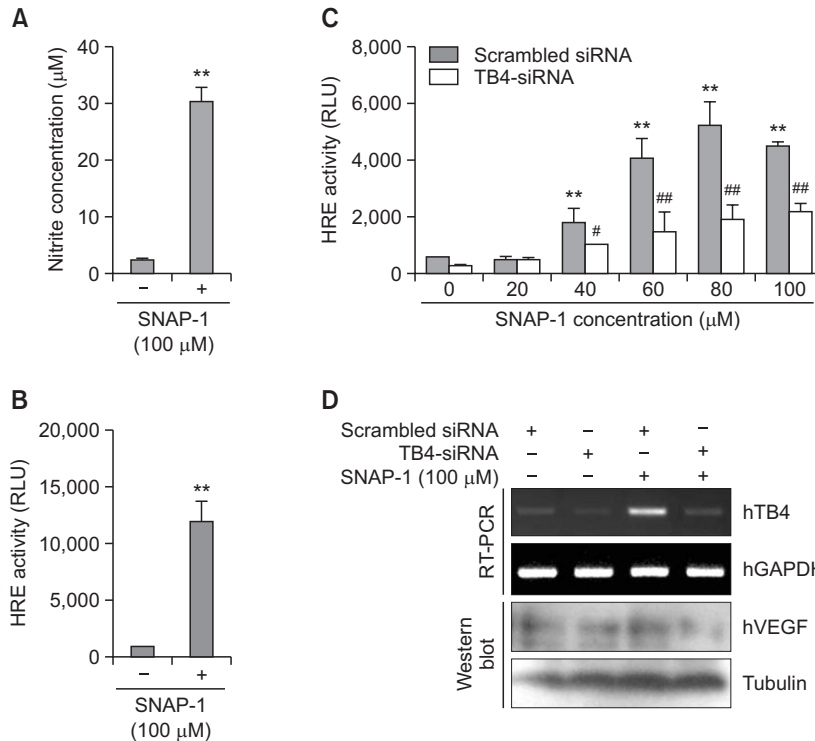
Fig. 2. VEGF expression is upregulated by SNAP-1, NO donor. (A) HeLa cells were treated with 100 μM SNAP-1. NO production was detected as nitrite accumulated in culture supernatant by using Griess reagents. (B) HeLa cells were transfected with pGL2 plasmid of hypoxia response element (HRE)-luciferase (Luc) and treated with 100 μM SNAP-1. Luc activity was measured with luminometer using Luc substrate. Data in bar graph represent mean ± SED. **p<0.01, statistical significance vs. SNAP-1-untreated group (A and B). (C-D) HeLa cells were co-transfected with Tβ4-siRNA and pGL2-HRE-Luc plasmid. Then, cells were treated with various concentrations of SNAP-1. Luc activity was measured with luminometer using Luc substrate. Data in bar graph represent mean ± SED. Luc activity was measured with luminometer using Luc substrate. Data in bar graph represent mean ± SED. **p<0.01, statistical significance vs. SNAP-1-untreated group. #*p*<0.05; ##*p*<0.01, statistical significance vs. scrambled siRNA-treated group at each concentration of SNAP-1 (C). RNA was purified with TRIZOL reagent as described in materials and methods. Tβ4 transcript level was measured by RT-PCR and VEGF levels were detected by western blot analysis (D).

duced by the treatment with L-NMMA under hypoxia condition (Fig. 3A). Hypoxia-induced increase in Tβ4 and HIF-1α was attenuated by the treatment with L-NMMA (Fig 3B). In addition, transcriptional and protein level of VEGF were also inhibited by the treatment with L-NMMA under hypoxia condition (Fig. 3B, C). Data demonstrate that hypoxia-inducible VEGF expression might be resulted from the regulation of NO production under hypoxia condition. It also suggests that VEGF expression could be controlled by NO-mediated Tβ4 expression and HIF-1α stabilization.

VEGF expression is restored by the transfection of pCMV-Tβ4 plasmids in L-NMMA-treated cells

To examine the role of Tβ4 on NO-dependent VEGF expression, Tβ4 was over-expressed by the transfection of pCMV-Tβ4 plasmids (Fig. 4A). VEGF protein level was increased by pCMV-Tβ4 transfection and then it was reduced by the treatment with L-NMMA. In contrast, L-NMMA-treated decrease in VEGF level was restored by the transfection of pCMV-Tβ4 plasmids (Fig. 4B). In addition, NO production was reduced by the treatment with L-NMMA and it was reversed by the transfection of pCMV-Tβ4 plasmids (Fig. 4C). These results evidenced that VEGF expression could be dependent on NO level. It suggests that NO-dependent VEGF expression might

be regulated by Tβ4 expression.

DISCUSSION

The increased HIF-1 activity provides a molecular basis for other adaptations of cancer cells to hypoxia that are critical for establishment of a primary tumor and its progression to the lethal phenotype (Semenza, 2000). VEGF plays a key role in angiogenesis and its gene transcription is activated by translocation of HIF-1 (Richard *et al*., 1999; Zhong *et al*., 1999). Hypoxic condition increases VEGF, Tβ4 expression (Gnecchi *et al*., 2006; Smart *et al*., 2007; Oh *et al*., 2008) and NO production (Maulik and Das, 2002). NO can promote tumor invasion and metastasis by activating various enzymes. VEGF expression is also upregulated by NO production, which lead to tumor angiogenesis (Xu *et al*., 2002; Fukumura *et al*., 2006; Hussain *et al*., 2008). Although it has been known that NO plays a key role in VEGF expression, it is required to define the mechanism of action or intermediate protein on NO-mediated VEGF expression. Here, we investigated whether NO-mediated VEGF expression under hypoxic condition is controlled by Tβ4 expression in HeLa cervical cancer cells. Our data showed that NO production and VEGF expression
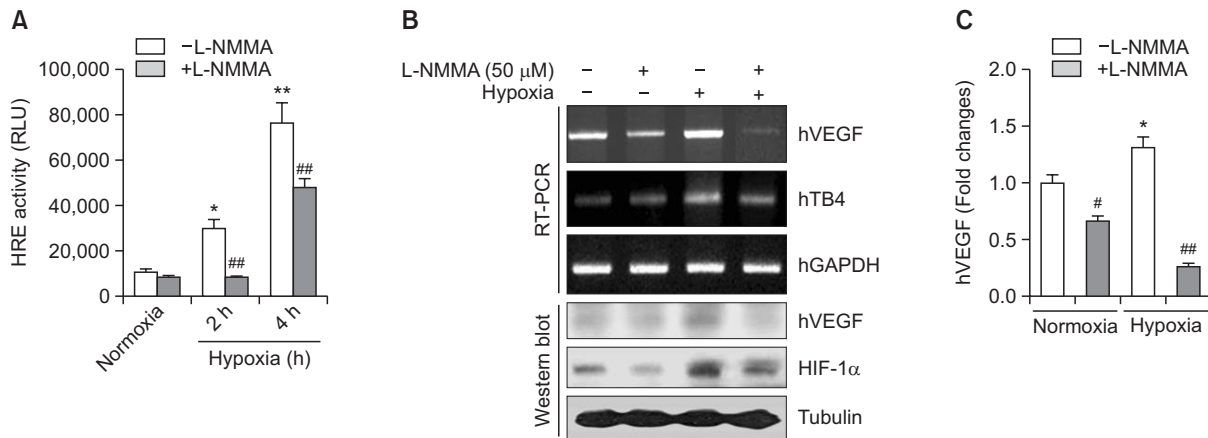
Fig. 3. L-NMMA, NOS inhibitor attenuated hypoxia-inducible VEGF expression. (A) HeLa cells were transfected with pGL2 plasmid of hypoxia response element (HRE)-luciferase (Luc) and incubated under normoxic or hypoxic condition in the presence or absence of L-NMMA. Luc activity was measured with luminometer using Luc substrate. Data in bar graph represent mean ± SED. *$p<0.05$; **$p<0.01$, statistical significance vs. normoxia control group. ##$p<0.01$, statistical significance vs. L-NMMA-untreated group at each time point under hypoxic condition. (B-C) HeLa cells were treated with L-NMMA and incubated under normoxic or hypoxic condition. RNA was purified with TRIZOL reagent. hVEGF and Tβ4 transcript level was measured by RT-PCR (B, top). hVEGF and HIF-1α in cell lysates were detected by western blot analysis (B, bottom). hVEGF transcripts were normalized and data in bar graph represent mean ± SED. *$p<0.05$, statistical significance vs. normoxia control group. #$p<0.05$; ##$p<0.01$, statistical significance vs. L-NMMA-untreated control at each normoxic or hypoxic condition (C).
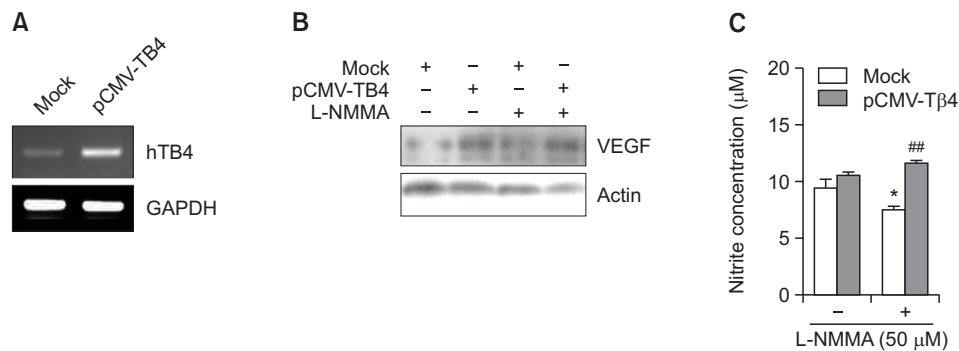

Fig. 4. VEGF expression reduced in L-NMMA-treated cells was restored by the transfection with pCMV-Tβ4 plasmid. (A-C) HeLa cells were transfected with control pCMV (Mock) or pCMV-Tβ4 plasmids. Then, HeLa cells were treated with L-NMMA and incubated under normoxic or hypoxic condition. RNA was purified with TRIZOL reagent. Tβ4 transcript level was measured by RT-PCR (A). Cells were incubated in the presence or absence of L-NMMA and cell lysates were prepared from HeLa cells transfected with Mock control or pCMV-Tβ4 plasmids. Then, hVEGF protein levels were detected by western blot analysis (B). NO production was detected as nitrite accumulated in culture supernatant by using Griess reagents. Data in bar graph represent mean ± SED. *$p<0.05$, statistical significance vs. L-NMMA-untreated control group. ##$p<0.01$, statistical significance vs. Mock control in L-NMMA-treated group (C).

was reduced by Tβ4-siRNA under hypoxic condition (Fig. 1). SANP-1, NO donor, increases HRE activity and the expression of VEGF (Fig. 2). VEGF expression reduced by L-NMMA, NOS inhibitor, is reversed by overexpression of Tβ4 (Fig. 3, 4). It suggests that NO-dependent VEGF expression is regulated via Tβ4 expression under hypoxic condition.

It is possible to suggest a few mechanism of action on Tβ4 expression. Since NO activate guanylate cyclase producing cGMP and protein kinase G (PKG) (Deguchi *et al*., 2004; Murad, 2006). So, cGMP or PKG could be involved in Tβ4 expression through the activation of transcription factor. Further study is required to define transcription factors to activate Tβ4 promoter. Another mechanism is direct activation of transcription factor by NO (Chamorro-Jorganes *et al*., 2011; Kanao *et al*., 2012), which might increase Tβ4 expression. HIF-1α also might be a candidate to bind Tβ4 promoter under hypoxic con-

dition. Since Tβ4 increased HIF-1α protein level (Oh *et al*., 2008) and the increase in HIF-1 activity is associated with a molecular adaptation of cancer cells to hypoxia (Semenza, 2000), positive feedback regulation might be involved in between Tβ4 expression and HIF-1α stabilization. Then, Tβ4 lead to HIF-1α stabilization and sequentially lead to an increase in Tβ4 expression by binding HIF-1α on Tβ4 promoter. It is also possible that nitric oxide synthase (NOS) expression could be regulated by HIF-1α acting on NOS promoter, which lead to NO production in hypoxia condition. These suggest that VEGF expression could be controlled by Tβ4 expression via NO production under hypoxic condition. However, it remains to be clarified 1) whether a Tβ4-binding element is present in the promoter of VEGF, 2) which sequence in the Tβ4 promoter binds NO-associated transcription factor or HIF-1α, and 3) the mechanism of action underlying Tβ4-regulated

VEGF expression.

Previous reports showed that various kinases except PKG could be activated by NO. NO and/or cGMP activate c-Src/PI3K- and PKG-dependent ERK 1/2 (Tejedo *et al*., 2004), and p21Ras-Raf-1 kinase-MEK-ERK1/2 (Oliveira *et al*., 2003). NO also activate CaMKII by an increase in Ca^{2+} leak from sarcoplasmic reticulum (Curran *et al*., 2014) So, it is possible to explain that hypoxia-inducible VEGF expression might be due to the participation of various signaling molecules including ERK in Tβ4-regulated NO production. Further study is required to define the main signaling molecules on Tβ4-mediated NO production.

Collectively, although it has not been defined whether VEGF promoter bind Tβ4 and what mechanism of action is involved in NO-mediated VEGF expression, hypoxia-inducible NO could influence the increase in VEGF expression through Tβ4. Data suggest that Tβ4 may participate in VEGF expression by a hypoxia-inducible NO.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The manuscript has been reviewed and approved by all authors. No potential conflicts of interest were disclosed.

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