Tat-Mediated p66shc Transduction Decreased Phosphorylation of Endothelial Nitric Oxide Synthase in Endothelial Cells

Sang Ki Lee¹,², Ji Young Lee¹, Hee Kyung Joo¹, Eun Jung Cho¹, Cuk Seong Kim¹, Sang Do Lee¹, Jin Bong Park¹, and Byeong Hwa Jeon¹

¹Infection Signaling Network Research Center, Research Institute of Medical Sciences, Department of Physiology, School of Medicine, Chungnam National University, Daejeon 301-131, ²Department of Sports Science, Chungnam National University, Daejeon 305-765, Korea

INTRODUCTION

Shc is an adaptor protein containing a C-terminal src homology collagen domain-2. The mammalian adaptor protein ShcA has three isoforms with relative molecular masses of 46, 52, and 66 kDa. p66shc possesses an N-terminal collagen-homology domain that is not present in p46shc or p52shc. p66shc is phosphorylated on serine 36 within the N-terminal domain in response to oxidative stress [1]. Several studies have shown that endothelial dysfunction by oxidative stress is central in the pathogenesis of vascular dysfunction and atherogenesis [2-4]. Recently, it was reported that the p66shc(−/−) mouse is a unique genetic model for increased resistance to oxidative stress and prolonged life span in mammals [5]. Therefore, p66shc might represent a molecular target for therapies against vascular diseases.

The phosphorylation of endothelial nitric oxide synthase (eNOS) in endothelial cells is pivotal in the defense against vascular inflammatory diseases [6] and atherosclerosis [7]. Elucidation of the role of p66shc adaptor protein on eNOS activity will contribute to our understanding of vasomotor physiology and the pathophysiology of endothelial dysfunction and could provide insights for new therapies, particularly in hypertension and atherosclerosis. Genetic deletion of p66shc prevents endothelial dysfunction [8,9]. RNAi-mediated down-regulation of endogenous p66shc leads to the activation of endothelial nitric oxide synthase at serine 1177 [10]. Furthermore, p66shc phosphorylation is closely related to oxidative stress in endothelial cells [11] or hypertensive animals such as coarctation of aorta [12].

Direct intracellular delivery of proteins has been difficult to achieve due primarily to the plasma membrane barrier, which effectively prevents macromolecule uptake by limiting passive entry. One approach to circumvent these problems is to use HIV Tat-mediated protein transduction [13]. Protein transduction domains (PTD) offer an exciting therapeutic opportunity to treat many diseases such as vascular inflammation [14,15]. Using a PTD-fused protein might also help us understand target protein signaling, as its cellular transduction is rapid.

Our aim was to evaluate the potential usefulness of the Tat-p66shc fusion protein on the eNOS phosphorylation in endothelial cells. We investigated the transduction of the full length human Tat-p66shc fusion protein in endothelial cells and the resulting biological activity on eNOS phosphorylation in cultured endothelial cells.

ABBREVIATIONS: NO, nitric oxide; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; PTD, protein transduction domains; TNF, tumor necrosis factor; IPTG, Isopropyl-thiogalactoside; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate.
METHODS

Cell culture and reagent

Mouse MS-1 endothelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's-modified Eagle medium (DMEM) with 10% fetal bovine serum, 10 μM penicillin, and 10 μg/ml streptomycin. Antibodies to Shc (SC-1695, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ser36-p66shc (CN566807, Calbiochem, La Jolla, CA, USA), eNOS (SC-654, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ser1199-eNOS (#9571, Cell Signaling Technology, Danvers, MA, USA), and β-actin (SC-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Horseradish peroxidase (HRP)-labeled anti-rabbit antibody was obtained from Pierce biotechnology (Rockford, IL USA). Ni-nitrilotriacetic acid Sepharose was purchased from Qiagen (Valencia, CA, USA). Isopropyl-β-thiogalactoside (IPTG) was purchased from USB (Cleveland, OH, USA).

Recombinant protein expression and purification

Tat-p66shc was generated by inserting p66shc cDNA into pTAT-2.1. The human p66shc was isolated from Xpress-tagged p66shc CDNA in pcDNA3/1/His A (Invitrogen, Carlsbad, CA, USA) [10] by PCR using the following two primers; the sense primer was 5'-CGG GAT CCC GGA ATT CGG CTT ATG ATC CTC C-3' (containing an EcoRI restriction site), and the antisense primer was 5'-CGA AGC TTT CAC AGT TTC CCG TCC ACA GG-3' (containing a HindIII restriction site). After digesting with EcoRI and HindIII, the full length p66shc constructs were cloned into the pTAT bacterial expression vector (pTAT-2.1, kindly donated by Steven Dowdy), which contains a six-histidine tag, for easy purification, p66shc (S636A) cDNA was kindly donated by Kaikobad Irani (Pittsburgh University). pTAT-p66shc plasmids were then transformed into the BL21 (DE3) strain of E. coli. Following 4 hours of induction with IPTG, the cells were sonicated in buffer Z (8 M urea, 100 mM NaCl, 20 mM HEPES) and recombinant proteins were purified on a Ni-NTA agarose column (Qiagen). After washing, Tat-p66shc was eluted using 250 mM imidazole containing Buffer Z followed by desalting on a PD-10 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to detect DCF-DA fluorescence. The cells were rinsed three times and incubated for 30 min with 10 μM DCF-DA at 37°C in Krebs-HEPES buffer and Hanks buffered salts solution, respectively. The absolute fluorescence of 20~25 random cells was quantified using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Superoxide production was assayed in the cultured endothelial cells using lucigenin chemiluminescence. In briefly, dark-adapted lucigenin solution (5 μM) was prepared in aerated Krebs-Hepes buffer. Cells were immersed in lucigenin solution and chemiluminescence detected with a Monolight software (Molecular Devices, Sunnyvale, CA, USA). The cells were cultured on chamber slides (2×10^5 cells/well) (Nalgen Nunc International, Rochester, NY, USA) to detect DCF-DA fluorescence. The cells were rinsed three times and incubated for 30 min with 10 μM DCF-DA at 37°C in Krebs-HEPES buffer and Hanks buffered salts solution, respectively. The absolute fluorescence of 20~25 random cells was quantified using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Superoxide production was assayed in the cultured endothelial cells using lucigenin chemiluminescence. In briefly, dark-adapted lucigenin solution (5 μM) was prepared in aerated Krebs-Hepes buffer. Cells were immersed in lucigenin solution and chemiluminescence detected with a Monolight luminometer. The chemiluminescence signal was integrated over 2 minutes.

Statistical analysis

Values are expressed as mean±SEM. The statistical evaluation was conducted a one-way analysis of variance followed by a Tukey’s post-hoc test, and p<0.05 was considered statistically significant.

RESULTS

Construction, expression, and purification of Tat-p66shc

First, we constructed the Tat-p66shc fusion protein using recombinant DNA technology. The Tat-p66shc expression vector contained a consecutive cDNA sequence encoding human p66shc, the Tat-PTD (RKKRRQRRR), and six histidine residues at the amino-terminus (Fig. 1). Tat-p66shc proteins were serially eluted with the treatment of 250 mM imidazole containing buffer Z. The purified Tat-p66shc proteins were confirmed by Comassie blue staining, which showed a molecular weight of approximately 70.5 kDa (Fig. 1).

Transduction of Tat-p66shc into cultured endothelial cells

Purified Tat-p66shc (30 nM) was added to cultured endothelial cells for various incubation times to evaluate the transduction ability in cultured endothelial cells. After the incubation, the cells were harvested and the change in transduced p66shc was analyzed with Western blotting using the anti-Shc antibody. As shown in Fig. 2A, the Tat-
p66shc fusion protein was detected in the cell lysates within 15 min and its transduction reached a maximum at 3 h. A significant level of transduced Tat-p66shc was present in the cells at 24 h (Fig. 2A). Moreover, Tat-p66shc fusion proteins were transduced into the cells in a dose-dependent manner in the range of 1~100 nM (Fig. 2B).

**Tat-p66shc protein transduction increased the superoxide production in the endothelial cells**

We next studied whether transduced Tat-p66shc affected hydrogen peroxide and superoxide production in cultured endothelial cells. Endothelial cells were incubated with Tat-p66shc, Tat-p66shc (S/A), p66shc, and Tat-GFP as a control for 3 h. After the incubation, hydrogen peroxide and superoxide production was measured in the endothelial cells using DCF-DA and lucigenin chemiluminescence. As shown in Fig. 3, Tat-p66shc (30 nM) for 3 h significantly elevated intracellular hydrogen peroxide compared with Tat-GFP and p66shc as control in endothelial cells. Also, the Tat-p66shc incubation (30 nM) increased superoxide production, as accessed by lucigenin chemiluminescence. However, Tat-p66shc-mediated ROS productions were greater than that of Tat-p66shc (S/A), suggesting important role of serine 36 residue of p66shc in ROS productions in endothelial cells.

**Tat-p66shc decreased endothelial nitric oxide synthase phosphorylation**

To understand the acute effect of p66shc on eNOS activity, we examined the effect of Tat-p66shc transduction on eNOS phosphorylation in cultured endothelial cells. In comparison to Tat-GFP treated cells, Tat-p66shc transduction did not affect eNOS protein expression; however, it decreased eNOS phosphorylation at the serine 1177 residue (Fig. 4A).

Finally, we investigated the role of Tat-p66shc protein transduction on eNOS phosphorylation in tumor necrosis factor (TNF)-α-stimulated endothelial cells. As shown in Fig. 4B, TNF-α increased p38 MAPK and eNOS phosphorylation. In contrast, Tat-p66shc protein transduction (30 nM for 3 h) augmented TNF-α-induced p38 MAPK phosphorylation and abrogated TNF-α-induced eNOS phosphorylation in endothelial cells.

**DISCUSSION**

The present study demonstrated that transduced Tat-p66shc fusion protein increased intracellular ROS production and inhibited eNOS phosphorylation in vascular endothelial cells.

Protein transduction domains (PTD) offer an exciting therapeutic opportunity to treat many diseases such as vas-
Fig. 3. Tat-p66shc transduction increased hydrogen peroxide and superoxide production in culture MS-1 endothelial cells. (A) Effect of Tat-p66shc on hydrogen peroxide production. Intracellular hydrogen peroxide production was evaluated using the peroxide-sensitive fluorophore 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). Control: p66shc protein alone, Tat-GFP (30 nM), Tat-p66shc (30 nM), Tat-p66shc S/A (30 nM). (B) Effect of Tat-p66shc on superoxide production. Intracellular superoxide production was evaluated using lucigenin chemiluminescence. Superoxide production levels are expressed as relative luminescence units, RLU/10⁶ cells. Control: p66shc protein alone, Tat-GFP (30 nM), Tat-p66shc (30 nM), Tat-p66shc S/A (30 nM). Each bar shows the mean±S.E. (n=5). NS, not significant, *p<0.05, *p<0.05 versus p66shc.

Fig. 4. Tat-p66shc transduction decreased endothelial nitric oxide synthase (eNOS) phosphorylation in culture MS-1 endothelial cells. (A) Effect of Tat-p66shc on basal eNOS phosphorylation. Tat-p66shc and Tat-GFP were incubated for 3 h with endothelial cells at the indicated concentrations, and then cell lysates were subjected to Western blot analysis. Note: Cells were not serum starved to detect basal eNOS phosphorylation. Tat-p66shc and Tat-GFP were incubated for 3 h with endothelial cells at the indicated concentrations, and then cell lysates were subjected to Western blot analysis. Note: Cells were not serum starved to detect basal eNOS phosphorylation. (B) Effect of Tat-p66shc on eNOS phosphorylation in the tumor necrosis factor (TNF)-α-stimulated endothelial cells for 0∼45 min. Tat-p66shc (30 nM) and Tat-GFP (30 nM) were incubated for 3 h, and then TNF-α was treated for the indicated times. The cells were serum starved for 18 h to reduce basal p38 MAPK and eNOS phosphorylation. This figure shows a representative experiment out of 3 made.
Tat-p66shc Inhibited eNOS Phosphorylation

P38 MAPK signaling pathway is implicated in the downregulation of eNOS phosphorylation, whereas p38 MAPK activation decreases eNOS activation, whereas p38 MAPK inhibitor treatment restores eNOS activity in endothelial cells [27,28]. Furthermore, activation of the p38 MAPK signaling pathway is implicated in the downregulation of eNOS activity [29]. In the present study, Tat-p66shc-mediated suppression of eNOS phosphorylation persisted even in the presence of TNF-α, suggesting that Tat-p66shc-mediated suppression of eNOS phosphorylation is regulated by p38 MAPK activation.

Taken together, these data show that the Tat-p66shc protein was efficiently transduced into cultured endothelial cells and suppressed eNOS phosphorylation in endothelial cells, suggesting that Tat-mediated p66shc transduction may be useful for suppressing eNOS activation in vascular pathogenesis.

ACKNOWLEDGEMENTS

We thank Dr. Steven Dowdy (University of California, San Diego, USA) for providing the Tat expression vector. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (2011-0006231, 2011-0016797 to B.H.J and 2011-0014483 to S.K.L.).

REFERENCES

1. Migliaccio E, Mele S, Saleini AE, Pelici G, Lai KM, Superti-Furga G, Possow T, Di Fiore PP, Lanfrancone I, Pelici PG. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J. 1997;16:706-716.

2. Stocker R, Keaney JF Jr. Role of oxidative modifications in atherosclerosis. Physiol Rev. 2004;94:1381-1478.

3. Molavi B, Mehta JL. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol. 2005;25:29-38.

4. Madamanchi NB, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol. 2005;25:29-38.

5. Napoli C, Martin-Padura I, de Nigris F, Giorgio M, Mansueto G, Sonnai P, Codorelli M, Sica G, De Rosa G, Pelici P. Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet. Proc Natl Acad Sci USA. 2003;100:2112-2116.

6. Kim CS, Son SJ, Kim EK, Kim SN, Yoo DG, Kim HS, Ryoo SW, Lee SD, Irani K, Jeon BH. Apurinic/apyrimidinic endonuclease/redox factor-1 inhibitor monocyte adhesion in endothelial cells. Cardiovasc Res. 2006;69:520-526.

7. Kawashima S. Malfunction of vascular control in lifestyle-related diseases: endothelial nitric oxide (NO) synthase/NO system in atherosclerosis. J Pharmacol Sci. 2004;96:411-419.

8. Martin-Padura I, de Nigris F, Migliaccio E, Mansueto G, Minardi S, Rienzo M, Lerman LO, Stendardo M, Giorgio M, De Rosa G, Pelici PG, Napoli C. p66shc deletion confers vascular protection in advanced atherosclerosis in hypercholesterolemic apolipoprotein E deficient mice. Endothelium. 2008;15:276-287.

9. Camici GG, Schiavioni M, Francia P, Bachschmid M, Martin-Padura I, Hershberger M, Tanner FC, Pelici P, Volpe M, Anversa P, Lüscher TF, Cosentino F. Genetic deletion of p66shc adaptors prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. Proc Natl Acad Sci USA. 2007;104:5217-5222.

10. Yamamori T, White AR, Mattagajashish I, Khanday FA, Haile A, Qi B, Jeon BH, Bugayenko A, Kasuno K, Berkowitz DE, Irani K. p66shc regulates endothelial NO production and endothelium-dependent vasorelaxation: implications for age-associated vascular dysfunction. J Mol Cell Cardiol. 2005;39:992-995.

11. Lee SK, Chung JI, Park MS, Joo HK, Lee EJ, Cho EJ, Park JB, Jeon BH. Alteration of p66shc is associated with endothelial dysfunction in the abdominal aortic coarctation of rats. FEBS Lett. 2008;582:2561-2566.

12. Becker-Hapak M, McAllister SS, Dowdy SF. Tat-mediated protein transduction into mammalian cells. Methods. 2001;24:177-186.

13. Song YJ, Lee YJ, Joo HK, Kim HS, Lee SK, Lee KH, Cho CH, Park JB, Jeon BH. Tat-APE1/ref-1 protein inhibits TNF-alpha-induced endothelial cell activation. Biochem Biophys Res Commun. 2008;368:68-73.

14. Lee YJ, Park KS, Cho EJ, Joo HK, Lee SK, Lee SD, Park JB, Chang SJ, Jeon BH. Human HOXA5 homeodomain enhances protein transduction and its application to vascular inflammation. Biochem Biophys Res Commun. 2011;410:312-316.

15. Anglewoc P, Deshpande SS, Qi B, Liu YX, Park YC, Jeon BH, Ozaki M, Irani K. Redox factor-1: an extra-nuclear role in the regulation of endothelial oxidative stress and apoptosis. Cell Death Differ. 2002;9:717-725.

16. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc Natl Acad Sci USA. 2000;97:13003-13008.

17. Francia P, delli Gatti C, Rachschmid M, Martin-Padura I, Savoia C, Migliaccio E, Pelici PG, Schiavioni M, Lüscher TF, Volpe M, Cosentino F. Deletion of p66shc gene protects against age-related endothelial dysfunction. Circulation. 2004;110:2889-2895.

18. Wu Z, Rogers B, Kachi S, Hackett SF, Sied A, Campochiaro PA. Reduction of p66shc suppresses oxidative damage in retinal pigmented epithelial cells and retina. J Cell Physiol. 2006;209:996-1005.

19. Anderson TJ. Nitric oxide, atherosclerosis and the clinical relevance of endothelial dysfunction. Heart Fail Rev. 2003;8:71-86.

20. Kim DD, Sánchez FA, Durán RG, Kanetaka T, Durán WN. Endothelial nitric oxide synthase is a molecular vascular target for the Chinese herb Danshen in hypertension. Am J Physiol Heart Circ Physiol. 2007;292:H2131-H2137.

21. Cho EJ, Park MS, Kim SS, Kang Y, Choi S, Lee YR, Chang SJ, Lee KH, Lee SD, Park JB, Jeon BH. Vasorelaxing activity of ulmus davidiana ethanol extracts in rats: activation of endothelial nitric oxide synthase. Korean J Physiol Pharmacol. 2011;15:339-344.

22. Shin W, Cuong TD, Lee JH, Min B, Jeon BH, Lim HK, Ryoo S. Arginase inhibition by ethylxacetate extract of caesalpinia sappan lignum contributes to activation of endothelial nitric oxide synthase. Korean J Physiol Pharmacol. 2011;15:123-128.

23. Lee SK, Kim YS, Kim CS, Son SJ, Yoo DG, Lee KH, Lee SD, Park JB, Jeon BH, p66shc adaptor protein suppresses the activation of endothelial nitric oxide synthase in mouse embryonic fibroblasts. Korean J Physiol Pharmacol. 2006;10:155-159.

24. Grethe S, Ares MP, Andersson T, Pärn-Ares MI. p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bel-7(L). Exp Cell Res. 2004;298:632-642.

25. Yu JH, Kim CS, Yoo DG, Song YJ, Joo HK, Kang G, Jo YJ, Park JB, Jeon BH. NADPH oxidase and mitochondrial ROS
are involved in the TNF-alpha-induced vascular cell adhesion molecule-1 and monocyte adhesion in cultured endothelial cells. Korean J Physiol Pharmacol. 2006;10:217-222.

27. Li G, Barrett Ed, Barrett MO, Cao W, Liu Z. Tumor necrosis factor-alpha induces insulin resistance in endothelial cells via a p38 mitogen-activated protein kinase-dependent pathway. Endocrinology. 2007;148:3356-3363.

28. Zhang XH, Yokoo H, Nishioka H, Fujii H, Matsuda N, Hayashi T, Hattori Y. Beneficial effect of the oligomerized polyphenol oligonol on high glucose-induced changes in eNOS phosphorylation and dephosphorylation in endothelial cells. Br J Pharmacol. 2010;159:928-938.

29. Xing F, Jiang Y, Liu J, Zhao K, Mo Y, Liu Z, Zeng Y. Downregulation of human endothelial nitric oxide synthase promoter activity by p38 mitogen-activated protein kinase activation. Biochem Cell Biol. 2006;84:780-788.