Korean J Physiol Pharmacol
Vol 10: 155-159, June, 2006

p66shc Adaptor Protein Suppresses the Activation of Endothelial Nitric Oxide Synthase in Mouse Embryonic Fibroblasts

Sang Ki Lee, Young Shin Kim, Cuk Seong Kim, Sook Jin Son, Dae Goon Yoo, Kwon Ho Lee, Sang Do Lee, Jin Bong Park, and Byeong Hwa Jeon

Department of Physiology, College of Medicine, Chungnam National University, Daejeon 301-131, Korea

Among the Shc proteins, p66shc is known to be related to oxidative stress responses and regulation of the production of reactive oxygen species (ROS). The present study was undertaken to investigate the role of p66shc on endothelial nitric oxide synthase (eNOS) activity in the mouse embryonic fibroblasts (MEFs). When wild type (WT) or p66shc (−/−) MEFs were transfected with full length of eNOS cDNA, the expression and activity of eNOS protein were higher in the p66shc (−/−) MEFs. These phenomena were reversed by reconstitution of p66shc cDNA transfection in the p66shc (−/−) MEFs. The basal superoxide production in the p66shc (−/−) MEFs was not significantly different from that of WT of MEFs. However, superoxide production induced by NADPH in the p66shc (−/−) MEF was lesser than that in WT MEFs. When compared with WT MEFs, cell lysate of p66shc (−/−) MEFs showed significantly increased H-ras activity without change of endogenous H-ras expression. Our findings suggest the pivotal role of p66shc adaptor protein played in inhibition of endothelial nitric oxide production via modulation of the expression and/or activity of eNOS protein.

Key Words: p66shc, Endothelium, Endothelial nitric oxide synthase, Nitric oxide

INTRODUCTION

Shc proteins are modular structure, and were first described as scaffolding proteins that bridge the growth factor bound protein (grb2)-son of sevenless (sos1) complex to phosphorylated receptor tyrosine kinases, resulting in activation of the membrane-bound GTPase ras (Li et al, 1993). Shc genes have been found in the mammalian system and Shc proteins are present in various tissues of human and mouse (Luzi et al, 2000).

The Shc gene encodes two mRNA species: p66shc and p46/p52shc. Structurally, p66shc possesses an N-terminal collagen-homology domain that is not present in p46shc and p52shc. Specific phosphorylatable residues within this domain are critical for the reactive oxygen species (ROS) regulating function of p66shc (Migliaccio et al, 1997).

p66shc plays a crucial role in the regulation of oxidative stress response and apoptosis. p66shc (−/−) mouse embryonic fibroblasts (MEFs) cells are resistant to producing ROS in response to nutrient deprivation or serum starvation. Overexpression of a wild-type p66shc transgene in normal MEFs resulted in increased sensitivity to apoptosis under the same conditions (Migliaccio et al, 1999), supporting the theory that p66shc might participate in the regulation of the aging process by regulating oxidant generation and inducing apoptosis (Migliaccio et al, 1999; Nemoto & Finkel, 2002).

Several studies have shown that endothelial dysfunction by oxidation stress is central in the pathogenesis of vascular dysfunction and atherogenesis (Molavi & Mehta, 2004; Stocker & Keaney, 2004; Madamanchi et al, 2005). Recently, p66shc (−/−) mouse has been reported to be the unique genetic model of increased resistance to oxidative stress and prolong life span in mammals (Napoli et al, 2003). Therefore, p66shc might represent a molecular target for therapies against vascular diseases.

Nitric oxide production by endothelial nitric oxide synthase (eNOS) in the endothelial cells exert pivotal defense function against vascular inflammation (Kim et al, 2006) or atherosclerosis (Kawashima, 2004). Elucidation of the effect of p66shc adaptor protein on eNOS activity and NO bioavailability is expected to contribute to our understanding of the physiology of vasomotion and the pathophysiology of endothelial dysfunction, and could provide insights for new therapies, particularly in hypertension and atherosclerosis. Recently, it has been reported that down-regulation of p66shc increased endothelium-dependent relaxation in mice (Yamamori et al, 2005). However, the role of p66shc on the eNOS activity and its underlying mechanism are not clearly understood. Therefore, in the

Corresponding to: Byeong Hwa Jeon, Department of Physiology, College of Medicine, Chungnam National University, 6 Munhwa-dong, Jung-gu, Daejeon 301-131, Korea. (Tel) 82-42-580-8214, (Fax) 82-42-585-8440, (E-mail) bhjeon@cnu.ac.kr
* Sang Ki Lee and Young Shin Kim : equally contributed as first authors.

ABBREVIATIONS: MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; NADPH, nicotinamide adenine dinucleotide phosphate.

present study, we investigated the role of p66shc on the eNOS enzyme activity, expression and nitric oxide (NO) in the p66shc (−/−) MEF and human endothelial cells.

METHODS

Cell culture

Spontaneously immortalized MEFs cells and p66shc (−/−) MEFs were gifts of Kaikobad Irani (University of Pittsburgh, USA). Cells were maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% bovine serum albumin.

Cell transfection and immunoblotting

Mouse embryonic fibroblasts were transfected with eNOS (1 μg) and p66shc plasmids (2 μg) using Lipofectamine 2000 (Invitrogen). All transfections were balanced for the amount of DNA using pcDNA3.1. After 24 hours of transfection with the DNA, expression and phosphorylation of the protein (50 μg) were determined by immunoblotting with antibodies to eNOS (SC-654, Santa Cruz), phosphoserine-1177 eNOS (#9571, Cell signaling), and H-ras (SC-520, Santa-Cruz).

Measurements of NO

Twenty four hours after transfection, media from cells were processed for measurement of NO intermediary metabolite nitrite (NO_2) and nitrate (NO_3), the stable breakdown product of NO, by specific light absorbance as per manufacture's recommendations (Calbiochem). Thus media were de-proteinized using a 10 kDa cufoff filter. Absorbance of media of cells was subtracted from the control for background levels of NO_2+ NO_3 found in media.

Measurement of superoxide

Dark-adapted lucigenin solution (5 μM) was prepared in aerated Krebs-HEPES buffer solution. Cell suspensions (10^5 cells) were immersed in lucigenin solution and chemiluminescence was detected with a Monolight luminometer, as described previously (Jeon et al, 2004). The chemiluminescence signals over 1 min were integrated. Freshly-prepared NADPH (100 μM) was added for 1 min prior to measurement of luminescence, where indicated.

Ras activity

Active (GTP-bound) H-ras in lysates of p66shc (−/−) MEF and WT of MEF cells was determined after 24 hours of transfection with an active ras pull-down assay using the ras-binding domain of raf-1 (UBI), according to the manufacture's recommendations. Total H-ras was measured in whole cell lysates: The densitometric values of the active and total-H-ras bands were quantified.

Statistical analysis

Values are expressed as mean±SEM. Statistical evaluation was performed using student-t test, with $p<0.05$ considered significant.

RESULTS

We first examined whether p66shc protein were expressed in the wild-type MEF and p66shc (−/−) MEFs. Under the basal condition, p46, p52, and p66shc proteins were found to be expressed in the WT MEF, whereas only p66shc protein was not expressed in the p66shc (−/−) MEF cells (Fig. 1).

In order to find out the role of p66shc for the nitric oxide production in the WT and p66shc (−/−) MEFs, nitric oxide metabolites (nitrite and nitrate) were measured with modified Griess reaction (Jeon et al, 2004) in the culture media of those MEFs which were transfected with plasmids, encoding bovine endothelial nitric oxide synthase cDNA and/or human p66shc cDNA, with lipofectamine 2000. Basal NO metabolites were significantly higher in the p66shc (−/−) MEFs relative to their WT MEFs (Fig. 2). To make sure that this change was due to loss of p66shc in the MEF cells, the effect of p66shc overexpression on

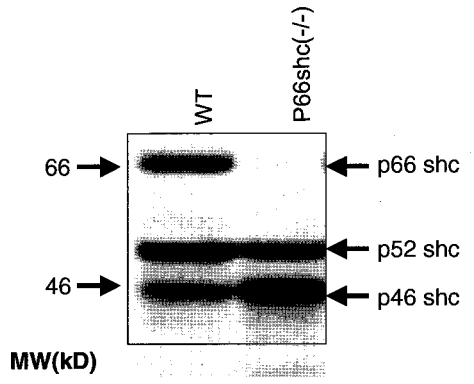

Fig. 1. Western blot analysis of Shc isoforms expression in wild type and p66shc (−/−) mouse embryonic fibroblasts (MEFs). Three types (46, 52, and 66 kda) of shc isoform were expressed in wild type (WT) MEFs, however only two types (46, 52 kda) of the isoform were expressed in p66shc (−/−) MEFs.

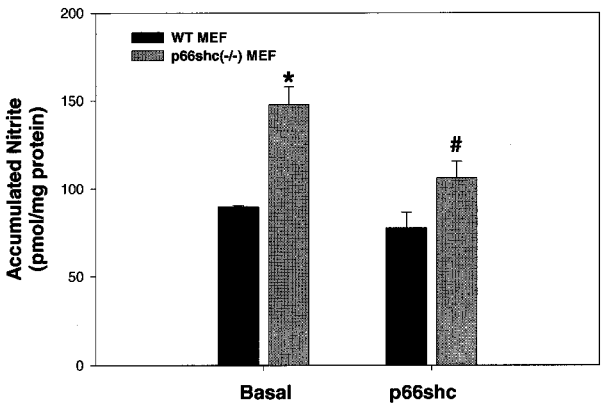

Fig. 2. Basal levels of accumulated NO_2 and NO_3 in the growth media of mouse embryonic fibroblasts transfected with endothelial nitric oxide synthase and/or p66shc cDNA. *$p<0.05$ compared to WT MEFs, #$p<0.05$ compared to basal cells.

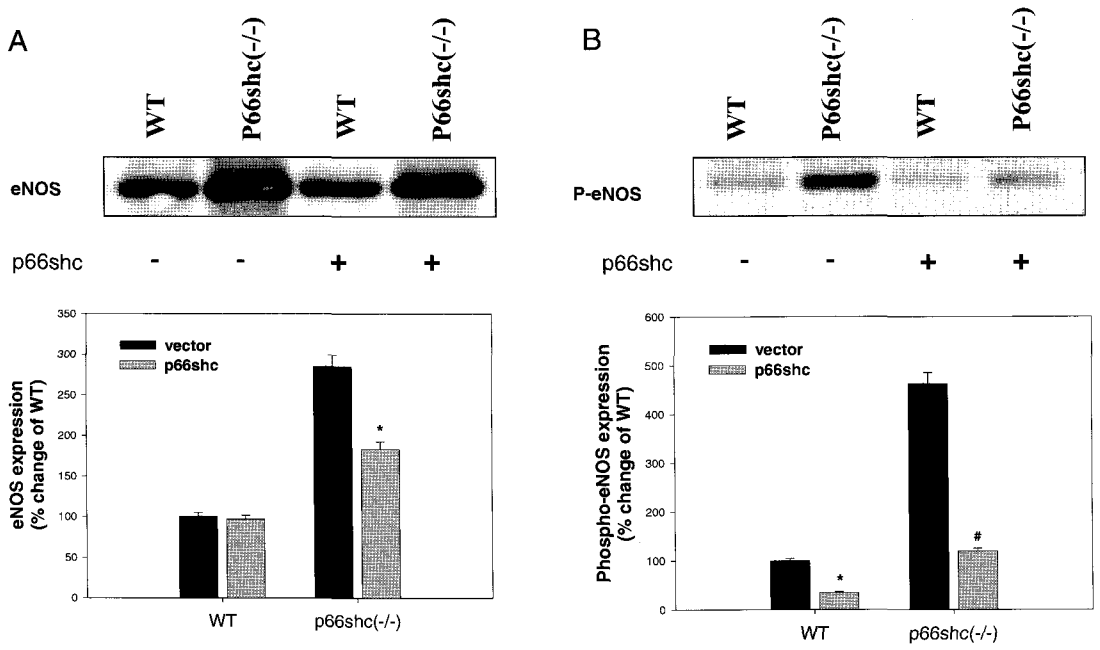

Fig. 3. p66shc decreased eNOS expression (A) and eNOS phosphorylation (B) in the WT and p66shc (−/−) of MEFs. Summarized data were plotted at bottom. eNOS expression represents densitometric values. The data are mean ±SEM for 3 separate experiments.

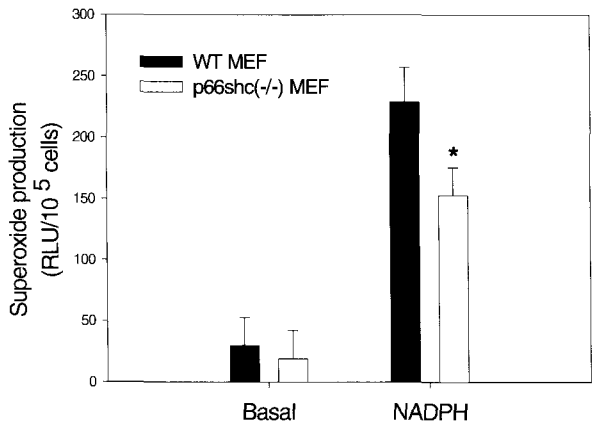

Fig. 4. Superoxide production in the p66shc (−/−) MEFs. Superoxide production was measured with the lucigenin method, as described in Material and Methods. To activate NADPH oxidase, NADPH (0.1 mM) was added to cells. Superoxide levels were calculated as the level per 10^5 cells. Each bar shows mean±SEM. *$p < 0.05$

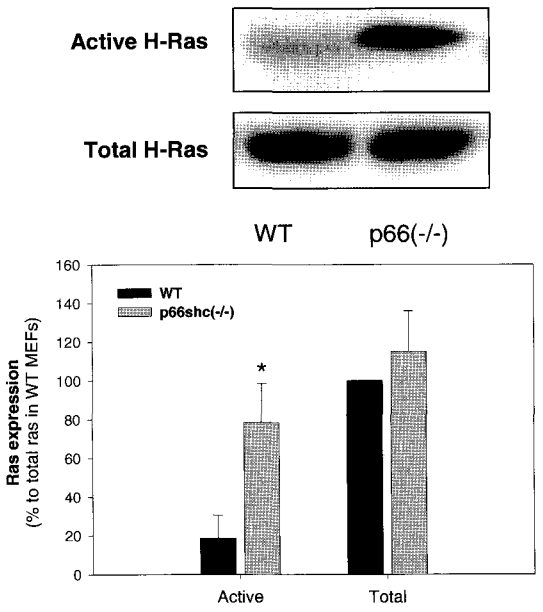

Fig. 5. Ras activation assay in the wild type and p66shc (−/−) MEF showed that active Ras was higher in p66shc (−/−) MEF than in WT. Shown is a representative experiment (A). Summarized data were plotted at bottom (B). *$p < 0.05$ compared to WT MEFs.

the NO metabolites was studied in eNOS-transfected p66shc (−/−) MEFs. As shown in Fig. 2, p66shc overexpression significantly suppressed NO production in the p66shc (−/−) MEF, compared to WT MEFs.

Having established the role of p66shc in the NO production, we then studied the effect of p66shc on the eNOS protein expression in the eNOS cDNA-transfected MEFs. When equal amounts of eNOS cDNA were transfected into WT and p66shc (−/−) MEFs, eNOS protein expression was higher in p66shc (−/−) MEF, compared to WT MEFs (Fig. 3A). Reconstitution of p66shc reduced the eNOS expression in the p66shc (−/−) MEF. To test whether p66shc adaptor protein was involved in the regulation of eNOS phosphorylation to augment basal NO production, we determined the change of eNOS phosphorylation in the p66shc (−/−) MEFs. As seen in Fig 3B, phosphorylation of eNOS on serine 1179 was markedly increased in the p66shc (−/−) MEFs, whereas phosphorylation of eNOS was reduced

by the transfection of p66shc.

To test whether p66shc (−/−) involved superoxide production via NADPH oxidase, we examined the superoxide level in the p66shc (−/−) MEFs using lucigenin chemiluminescence assay (Fig. 4). The basal superoxide production in p66shc (−/−) MEFs was not significantly different from that of WT MEFs. When 0.1 mM NADPH was added to the medium (as a substrate of NADPH oxidase), superoxide production was increased in both MEFs. However, superoxide production induced by NADPH in p66shc (−/−) MEF was less than that in WT MEFs.

Since the proto-oncogene H-ras lies upstream of PI3K and Akt kinase in insulin and EGF signalings, we wondered whether p66shc-regulated eNOS activity was dependent on H-ras. Therefore, we determined the effect of loss of p66shc on the endogenous H-ras activity and expression in the WT and p66shc (−/−) MEFs. Compared with WT MEFs, the cell lysate of p66shc (−/−) MEFs showed significantly increased H-ras activity without a change of endogenous H-ras expression (Fig. 5).

DISCUSSION

In the present study, we demonstrated a novel effect of p66shc on the regulation of eNOS activity and eNOS expression. Loss of p66shc protein increased eNOS activity and expression and decreased oxidative stress. The up-regulation of eNOS activity by the loss of p66shc might have been due to the up-regulation of H-ras dependent eNOS phosphorylation in the endothelial cells.

Regulation of eNOS activity is complex, and rapidly regulated through reversible non-covalent, calcium-dependent calmodulin binding, however, additional other mechanisms are also additionally involved, including induction by shear stress sensors, post-translational caveoli targeting, and serine, threonine and/or tyrosine phosphorylation (Robinson et al, 1995; Robinson & Michel, 1995; Fleming & Busse, 1999; Shaul, 2002). These data highlight the central role of eNOS played in maintenance of vascular tone, circulatory physiology and blood pressure, representing the integration of several stimulatory signals.

In the present study, eNOS activity and eNOS phosphorylation were found to be increased in the p66shc (−/−) MEFs. Furthermore, exogenous p66shc gene overexpression decreased nitric oxide production via suppression of eNOS activity in the MEF. Taken together, these observations strongly suggest that the modulation of post-transcriptional activity may be the key of p66shc in the control of eNOS activity. When p66shc and eNOS were co-overexpressed in the MEFs, eNOS protein expression was decreased by the p66shc. Transcriptional activity in overexpression system with plasmids was not affected by other factors, because plasmid has it's own exogenous strong promoter and transcripts it in a host-independent manner. Therefore, this suggests that p66shc may induce the degradation of eNOS protein. Recently, it has been reported that endothelium-dependent relaxation in response to acetylcholine was age-dependently impaired in WT mice, but not in p66shc (−/−) mice (Francia et al, 2004), a inactivation of the p66shc gene protects animals from age-dependent, ROS-mediated endothelial dysfunction, and diabetes (Napoli et al, 2003; Francia et al, 2004; Pagnin et al, 2005). These results are in support of our data that p66shc is a regulator of nitric oxide signal transduction pathway which is relevant to endothelial function.

All three Shc proteins (p46, p52 and p66) participate in mitogenic signaling and oncogenesis by regulating receptor tyrosine kinase signaling. Although the SH2 domain of Shc is important for certain receptor interactions, such as epidermal growth factor (EGF) receptor and ErbB-2, the PTB domain can bind to phospholipids, implying a role of phosphatidylinositol 3-kinase (PI3K) in activation of Shc (Bonfini et al, 1996; Okada et al, 1997; Ugi et al, 2002). Tyrosine phosphorylation of p46 and p52 enables these shc proteins to bind to the adaptor Grb2 protein, which then recruits the guanine nucleotide exchange factor SOS, thereby activating Ras and subsequently the mitogen-activated protein kinase (MAPK) cascade (Bonfini et al, 1996; Migliaccio et al, 1997; Okada et al, 1997). Grb2 has been known to activate the Ras signaling pathway without interacting with p46 or p52shc. The participation of p46 or p52shc is therefore thought to enhance certain weak signals from growth factor receptors or G-protein coupled receptors (Okada et al, 1997; Foschi et al, 2001). No evidence has yet been presented to indicate that p66shc activates the Ras signaling pathway (Migliaccio et al, 1997). In the present study, we showed that H-ras activity was markedly increased in the p66shc (−/−) MEFs. This finding suggests that p66shc competes with p46 or p52shc for Grb2 binding (Okada et al, 1997), and also that p66shc serves as a dominant negative regulator of p46shc or p52shc-mediated Ras signaling.

In summary, our data revealed a hither-to-unrecognized role of p66shc in governing endothelial NO production and NOS activity. Exploration of the mechanism of p66shc adaptor protein in the modulation of eNOS activity may contribute to the finding of a strategy to control vascular function and vascular disorder.

ACKNOWLEDGEMENT

This work was supported by a grant from the Basic Research Program (R01-2004-000-10045-0) of Korea Science & Engineering Foundation and partly supported by research fund of Chungnam National University in 2004. We thank Dr. Kaikobad Irani who kindly donated p66shc (−/−) MEFs.

REFERENCES

Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L, Pelicci PG. Not all Shc's roads lead to Ras. *Trends Biochem Sci* 21: 257−261, 1996

Fleming I, Busse R. Signal transduction of eNOS activation. *Cardiovasc Res* 43: 532−541, 1999

Foschi M, Franchi F, Han J, La Villa G, Sorokin A. Endothelin-1 induces serine phosphorylation of the adaptor protein p66Shc and its association with 14-3-3 protein in glomerular mesangial cells. *J Biol Chem* 276: 26640−26647, 2001

Francia P, delli Gatti C, Bachschmid M, Martin-Padura I, Savoia C, Migliaccio E, Pelicci PG, Schiavoni M, Luscher TF, Volpe M, Cosentino F. Deletion of p66shc gene protects against age-related endothelial dysfunction. *Circulation* 110: 2889−2895, 2004

Jeon BH, Gupta G, Park YC, Qi B, Haile A, Khanday FA, Liu YX, Kim JM, Ozaki M, White AR, Berkowitz DE, Irani K. Apurinic/apyrimidinic endonuclease 1 regulates endothelial NO production and vascular tone. *Circ Res* 95: 902−910, 2004

Kawashima S. Malfunction of vascular control in lifestyle-related

diseases: endothelial nitric oxide (NO) synthase/NO system in atherosclerosis. *J Pharmacol Sci* 96: 411–419, 2004
Kim CS, Son SJ, Kim EK, Kim SN, Yoo DG, Kim HS, Ryoo SW, Lee SD, Irani K, Jeon BH. Apurinic/apyrimidinic endonuclease1/redox factor-1 inhibits monocyte adhesion in endothelial cells. *Cardiovasc Res* 69: 520–526, 2006
Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B, Schlessinger J. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* 363: 85–88, 1993
Luzi L, Confalonieri S, Di Fiore PP, Pelicci PG. Evolution of Shc functions from nematode to human. *Curr Opin Genet Dev* 10: 668–674, 2000
Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol* 25: 29–38, 2005
Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG. The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 402: 309–313, 1999
Migliaccio E, Mele S, Salcini AE, Pelicci G, Lai KM, Superti-Furga G, Pawson T, Di Fiore PP, Lanfrancone L, Pelicci PG. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. *Embo J* 16: 706–716, 1997
Molavi B, Mehta JL. Oxidative stress in cardiovascular disease: molecular basis of its deleterious effects, its detection, and therapeutic considerations. *Curr Opin Cardiol* 19: 488–493, 2004
Napoli C, Martin-Padura I, de Nigris F, Giorgio M, Mansueto G, Somma P, Condorelli M, Sica G, De Rosa G, Pelicci 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* 100: 2112–2116, 2003
Nemoto S, Finkel T. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science* 295: 2450–2452, 2002
Okada S, Kao AW, Ceresa BP, Blaikie P, Margolis B, Pessin JE. The 66-kDa Shc isoform is a negative regulator of the epidermal growth factor-stimulated mitogen-activated protein kinase pathway. *J Biol Chem* 272: 28042–28049, 1997
Pagnin E, Fadini G, de Toni R, Tiengo A, Calo L, Avogaro A. Diabetes induces p66shc gene expression in human peripheral blood mononuclear cells: relationship to oxidative stress. *J Clin Endocrinol Metab* 90: 1130–1136, 2005
Robinson LJ, Busconi L, Michel T. Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J Biol Chem* 270: 995–998, 1995
Robinson LJ, Michel T. Mutagenesis of palmitoylation sites in endothelial nitric oxide synthase identifies a novel motif for dual acylation and subcellular targeting. *Proc Natl Acad Sci USA* 92: 11776–11780, 1995
Shaul PW. Regulation of endothelial nitric oxide synthase: location, location, location. *Annu Rev Physiol* 64: 749–774, 2002
Stocker R, Keaney JF Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev* 84: 1381–1478, 2004
Ugi S, Sharma PM, Ricketts W, Imamura T, Olefsky JM. Phosphatidylinositol 3-kinase is required for insulin-stimulated tyrosine phosphorylation of Shc in 3T3-L1 adipocytes. *J Biol Chem* 277: 18592–18597, 2002
Yamamori T, White AR, Mattagajasingh 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* 39: 992–995, 2005