P53 Impairs Endothelium-Dependent Vasomotor Function Through Transcriptional Upregulation of P66shc

Cuk-Seong Kim, Saet-Byel Jung, Asma Naqvi, Timothy A. Hoffman, Jeremy DeRicco, Tohru Yamamori, Marsha P. Cole, Byeong-Hwa Jeon, Kaikobad Irani

Abstract—The transcription factor, p53, and the adaptor protein, p66shc, both play essential roles in promoting oxidative stress in the vascular system. However, the relationship between the two in the context of endothelium-dependent vascular tone is unknown. Here, we report a novel, evolutionarily conserved, p53-mediated transcriptional mechanism that regulates p66shc expression and identify p53 as an important determinant of endothelium-dependent vasomotor function. We provide evidence of a p53 response element in the promoter of p66shc and show that angiotensin II-induced upregulation of p66shc in endothelial cells is dependent on p53. In addition, we demonstrate that downregulation of p66shc expression, as well as inhibition of p53 function in mice, mitigates angiotensin II-induced impairment of endothelium-dependent vasorelaxation, decrease in bioavailable nitric oxide, and hypertension. These findings reveal a novel p53-dependent transcriptional mechanism for the regulation of p66shc expression that is operative in the vascular endothelium and suggest that this mechanism is important in impairing endothelium-dependent vascular relaxation. (Circ Res. 2008;103:1441-1450.)

Key Words: tumor suppressor p53 ■ p66shc angiotensin II ■ endothelial dysfunction

P66shc belongs to the shcA family of adaptor proteins. P66shc is structurally and functionally distinct from p52shc and p46shc, the other 2 members of this family. It has a unique N-terminal collagen homology (CH2) domain that is important in governing its activity, and it functions as a protein that promotes oxidative stress within cells and tissues. Cells lacking p66shc have reduced levels of oxidants, and mice deficient for p66shc are resistant to oxidative stresses, and have an extended lifespan.1,2 In addition to living longer, p66shc-deficient mice are protected against age-associated vascular disorders that are not necessarily associated with atherosclerosis, is highly controversial. P53 is undetectable in normal vascular specimens, but is expressed in endothelial cells, smooth muscle cells, and macrophages of advanced human atherosclerosis.7 In animal models of high-fat diet–induced atheroma formation, knockout of p53 results in an increase in aortic plaque area, increased rates of cell proliferation, and reduced rates of apoptosis in both monocytes/macrophages and smooth muscle cells in the plaque.5,9 In contrast, an extra allele of p53 does not protect against diet-induced atherosclerosis in mice.10 Adding to this controversy, there is little information regarding whether and how p53 impacts on endothelium-dependent vasorelaxation, and impairment of such relaxation seen in early atherosclerotic disease. There is some evidence that p53 may affect vascular reactivity, independent of atheroma formation. Pharmacological inhibition of p53 function reduces cerebral artery vasospasm after experimentally induced subarachnoid hemorrhage.11

P53 upregulates p66shc expression, and p66shc is indispensable for p53-induced apoptosis.12 However, the precise nature of the relationship between these 2 proteins has not been examined to date. In particular, whether p66shc is a transcriptional target of p53 and whether p53 plays a role in impairment of endothelium-dependent vasorelaxation are not known. Moreover, although there is clear evidence that p66shc mediates age-associated impairment of endothelial...
function, its contribution to changes in vasomotor function in response to physiological regulators of vascular tone is not clear. Hence, we investigated whether both p53 and p66shc play essential roles in impairing endothelium-dependent vasorelaxation triggered by angiotensin (Ang) II and whether transcriptional upregulation of p66shc by p53 could be an underlying mechanism for this effect.

Figure 1. Endothelial p66shc expression is regulated by p53. A and B, Overexpression of p53 increases endothelial p66shc expression. Human aortic endothelial cells were infected with an adenovirus encoding p53 (Adp53) or the inert E coli LacZ gene (AdLacZ) for 24 hours. P66shc RNA (A) and p66shc and p53 protein (B) were assessed by real-time PCR and immunoblotting, respectively. RNA was normalized to the GAPDH internal control and expressed relative to AdLacZ-infected cells. *P<0.05 compared with AdLacZ-infected cells. C, P53 mediates Ang II–induced p66shc upregulation in endothelial cells. Human umbilical vein endothelial cells (HUVECs) were challenged with Ang II. Endogenous p53 activity was inhibited by treatment with pifithrin-α for 1 hour. Control cells were treated with vehicle. Immunoblots for shcA, p53, and β-actin on whole cell lysates were performed at the indicated times. The graphs show quantification of p66shc and p53 from 3 independent experiments. Values are normalized for loading control (β-actin) and expressed relative to time 0. *P<0.05, **P<0.01 compared with time 0. D, Ang II increases p66shc expression in the vascular wall in vivo. Aortic rings from mice infused with Ang II (b) or vehicle (a) for 14 days were immunostained with p66shc-specific antibody. Dark brown staining is positive for p66shc. Note the increase in medial thickness in Ang II–treated mice. E, P66shc mediates p53-induced oxidative stress. HUVECs were coinfected with the indicated adenoviruses, and hydrogen peroxide levels were measured. **P<0.01 and #P>0.05 compared with AdLacZ alone. Immunoblot showing specific knockdown of p66shc in Adp66shcRNAi-infected HUVECs is shown (bottom).

Materials and Methods

Cells and Transfections

Human umbilical vein and human aortic endothelial cells were purchased from Clonetics (San Diego, Calif) and Cell Applications Inc (San Diego, Calif). Human embryonic kidney (HEK 293) cells were purchased from American Type Culture Collection. Cells were transfected with cDNA and reporter plasmids using Lipo-
fectamine 2000 (Invitrogen) per the recommendations of the manufacturer.

Animals

The p66shcRNAi transgenic mice were created in a B6SJL background. The transgene consists of a short hairpin sequence `5'-TGA GTC TCT GTC ATC GCT G TTC AAG AGA C AGC GAT GAC-3' directed to nucleotides 239 to 257 of p66shc RNA, transcribed by the U6 RNA polymerase III promoter. Nucleotides 239 to 257 in p66shc RNA are in the coding region for the CH2 domain, are conserved in human and mouse, and are not found in p46/52shc RNA. Mice were genotyped for presence of the transgene in tail genomic DNA by PCR. Several generations of the mice showed robust germline transmission of the transgene in tail genomic DNA by PCR. Several generations of the mice showed robust germline transmission of the transgene (data not shown). Age-matched wild-type littermates were used as controls. Osmotic minipumps (Alzet 2002 osmotic minipump, Alza, Palo Alto, Calif) were implanted subcutaneously in the interscapular area of mice anesthetized with 1% to 3% isoflurane. The pumps contained either vehicle solution (0.01 mol/L acetic acid in saline) or Ang II at a delivery rate of 1.44 mg/kg per day for 14 days. Where specified, wild-type mice implanted with osmotic minipumps were injected with pifithrin-α (2.2 mg/kg diluted in saline) or saline control intraperitoneally every 48 hours for 14 days. All animals were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Antibodies, Immunoprecipitations, and Immunoblotting

Anti-shcA (Becton Dickinson) and anti-p53 (Santa Cruz Biotechnology) antibodies were purchased. Immunoprecipitations of p53 were carried out by incubating 2 g of p53 antibody (Cell Signaling, IC12) with 1 mg of cell lysate overnight, followed by 40 μL of protein A–sepharose slurry (Amersham) for 4 hours. After washing, immunoprecipitates were boiled in SDS-PAGE loading buffer, subjected to SDS-PAGE, transferred to nitrocellulose filter, and probed with the specified primary antibody and the appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotech). Western blotting of 50 μg of whole cell lysates was similarly performed using appropriate primary and secondary antibodies. Chemiluminescent signal was developed using Super Signal West Pico or Femto substrate (Pierce), and blots were imaged and quantified with a Gel Doc 2000 Chemi Doc system with Quantity One software (Bio-Rad).

Site-Directed Mutagenesis

Mutagenesis was performed with commercially available Quick-Change kits (Stratagene). All mutations were verified by sequencing. Deletion constructs were made using appropriate restriction endonucleases.
Figure 3. P53 binds to the putative p53 binding sequence in the human p66shc promoter. A. The putative p53 binding sequence in the human p66shc promoter binds to p53 in vitro. Electrophoretic mobility-shift assay using HEP 293 cell lysate overexpressing p53 and a radiolabeled oligonucleotide (GAA CAA GCC GTG ACC AGC ATG TCC T) with a sequence corresponding to the putative p53 binding site in the human p66shc promoter. Lane 1 shows free radiolabeled oligonucleotide; lane 2, radiolabeled oligonucleotide with cell lysate; lane 3, radiolabeled oligonucleotide with cell lysate and excess unlabeled oligonucleotide; lane 4, radiolabeled oligonucleotide with cell lysate and excess unlabeled mutant oligonucleotide (GAA CAG GCC GTG ACC AGG TCC T) [bold letters indicate mutated base pairs]; lane 5, radiolabeled oligonucleotide with cell lysate and p53 antibody. *P53-oligonucleotide complex. B. p53 binding sequence in intron 1 binds to the putative p53 binding sequence in the in intron 1 of the human shc1 gene. Chromatin immunoprecipitation assay in HEK 293 cells overexpressing p53. Immunoprecipitated p53 (lanes 2 and 4) or immunoprecipitates of nonimmune IgG (lanes 1 and 3) were used as the template for amplification of a 129-bp region (arrow) in intron 1 of the human shc1 gene (lanes 1 and 2) or a 177-bp region in the human GAPDH gene (lanes 3 and 4). The 129-bp region in the shc1 gene encompasses the putative p53 binding site of the p66shc promoter. Bands in all lanes below the 129-bp amplified product represent primer–dimer pairs. Pull-down of p53 in p53 and nonimmune IgG immunoprecipitates is shown (bottom).

Promoter Reporter Assays
A 1141-bp fragment of the human p66shc promoter region, −1096 to +44 base pairs relative to the ATG start codon (encompassing the putative p53 response element) was amplified from genomic DNA of HEP 293 cells, using appropriate primers. The fragment was cloned into the pGL4.1 firefly luciferase reporter vector (Promega). The mutant and Δp53 reporter plasmids were constructed by site-directed and deletion mutagenesis. The promoter–reporter constructs were cotransfected with a Renilla luciferase plasmid. Firefly and Renilla luciferase luminescence were measured using the Dual Luciferase reporter kit (Promega) according to the recommendations of the manufacturer. The firefly/Renilla ratio was calculated to normalize for variations in transfection efficiencies.

Electrophoretic Mobility-Shift Assay
Ten micrograms of cell extract were incubated with 105 cpm of a 32P-labeled oligonucleotide 5′-GAA CAA GCC GTG ACC AGC ATG TCC T-3′ (corresponding to the 25-bp putative p53-binding element in the human p66shc promoter in intron 1 of the SHC1 gene) for 15 minutes in binding buffer (10 mmol/L Tris, pH 7.4, 80 mmol/L KCl, 5% glycerol, 1 mmol/L DTT, 0.25 μg of dl-dC) at room temperature. Where indicated, 1 μg of p53 antibody (Santa Cruz Biotechnology, SC-98, 1801X), a 10-fold excess of unlabeled mutant oligonucleotide, or a 10-fold excess of unlabeled oligonucleotide mutated at 2 nucleotides (5′-GAA CAG GCC GTG ACC AGG TCC T-3′) was added to the mixture. Incubation mixtures were run out on a 6% polyacrylamide gel and autoradiographed.

Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation assays were performed in HEP 293 cells transfected with p53 using a ChIP Assay Kit (Upstate) and a rabbit polyclonal antibody for p53 (Santa Cruz Biotechnology, FL-393). Control immunoprecipitations were carried out with nonimmune rabbit IgG. Primers (forward 5′-GAG CCA TCG GTG GTG TGG TGG-3′, reverse 5′-GAA TAA AGT TCA ACC TGG ATG-3′) were used to PCR amplify a 129-bp fragment of human genomic DNA in intron 1 of the SHC1 gene from p53 and nonimmune IgG immunoprecipitates. This fragment encompasses the putative p53 binding site. As an additional negative control, PCR amplification was performed for a 177-bp region of the human GAPDH gene using primers (forward, 5′-ATG ACA TCA AGA AGG TGG TG-3′; reverse, 5′-CAT ACC AGG AAA TGA GCT GTG-3′), p53 immunoprecipitates as the template, and identical cycling conditions.

Hydrogen Peroxide Measurement
Hydrogen peroxide levels in live cells was measured using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes) as previously described.13

Real-Time PCR
Total RNA from cells or thoracic aorta of mice was isolated by the acid guanidinium thiocyanate/phenol/chloroform method. Real-time PCR was performed using the Prism 7000 Sequence Detection System (Applied Biosystems) with the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen). The primer sequences for human p66shc are: forward, 5′-AAG TAC AATCCA CTC CGG AAT GA-3′; reverse, 5′-GG GCC CCA GGG ATG AAG-3′. The primer sequences for mouse p66shc are: forward, 5′-GAT AGT CCG ACT ACC TGT TGT-3′; reverse, 5′-CAT AGG AAA TGA GCT GTG-3′. The ampiccons generated using these human and mouse primers are within the coding region of the unique CH2 domain of p66shc. Human and mouse GAPDH was used as internal controls. The primer sequences for human GAPDH are: forward, 5′-ATG ACA TCA AGA AGG TGG TG-3′; reverse, 5′-CAT ACC AGG AAA ATG AGC TTG-3′. The primer sequences for mouse GAPDH are: forward, 5′-GGC AAA TTA AAC GGC ACA GT-3′; reverse, 5′-GCC TCC TGG AAG ATG GTG AT-3′. Dissociation curves were monitored to check the aberrant formation of primer–dimers.
Adenoviral Constructs and Ex Vivo Infections
Recombinant adenoviruses encoding wild-type p53 (Adp53) and Escherichia coli LacZ (AdLacZ) were obtained from the Vector Core, University of Pittsburgh. The Adp66shcRNAi virus has been previously described.5 Aortic rings from 3- to 4-month-old Wistar–Kyoto rats were incubated with the specified adenovirus ex vivo for 24 hours as described previously14 before measurement of vascular reactivity.

Histology
Deparaffinized rat and mouse aortic ring sections were permeabilized and processed using a Vectastain Universal Quick Kit (Vector Laboratories, PK-8800). Primary antibody to p53 (Santa Cruz Biotechnology) and p66shc (UBI, 07-150) were used at a 1:50 dilution, followed by biotinylated secondary antibody, streptavidin peroxidase solution, DAB peroxidase substrate, and hematoxylin counterstain. Sections were digitally imaged with a Zeiss Axiovert 200 microscope.

Vascular Reactivity Measurements
Endothelium-dependent and endothelium-independent vasorelaxation and bioavailable NO were measured in aortic rings of 8- to 12-week-old mice and rats as described previously.14 Endothelial denudation of aortic rings was performed by rubbing the intimal surface of the aorta with a fine forceps.

Blood Pressure Measurements
Blood pressure in live mice was recorded by the tail cuff method. Mice were restrained in a cylindrical restrainer for 10 minutes to acclimatize them to the apparatus (Noninvasive Blood Pressure Monitor NIBP8, Columbus Instruments) before actual blood pressure and heart rate recordings are made. Ten blood pressure and heart rate recordings over a period of 10 minutes were obtained and averaged.

Results
To examine the role of p53 in regulating p66shc expression, we first forced expression of p53 in vascular endothelial cells. Adenoviral-induced increase in expression of p53 in endothelial cells resulted in an increase in p66shc mRNA and protein (Figure 1A and 1B). Because p66shc mediates myocardial damage triggered by the biologically relevant vasoactive agent Ang II,15 we investigated the effect of Ang II on p66shc expression in endothelial cells. Endothelial cells challenged with Ang II displayed a robust increase in p66shc expression (Figure 1C). In addition, aortas of mice challenged with Ang II infusion showed a significant upregulation of p66shc expression, primarily in the endothelium and the adventitia (Figure 1D). To determine the role of endogenous p53 in this upregulation of p66shc expression, we next treated the cells with pifithrin-α, an inhibitor of p53 function in the context of Ang II challenge. Ang II–induced increase in p66shc expression was completely abolished by treatment with pifithrin-α (Figure 1C). Ang II also upregulated p53 expression, which was also abrogated by pifithrin-α, consistent with the role of p53 in regulating its own expression.17 A similar increase in p53 expression was observed in rat aortic endothelial cells (Figure I in the online data supplement, available at http://circres.ahajournal.org), and basal p53 and p66shc expression was also diminished in cells treated with...
Figure 5. Knockdown of p66shc or inhibition of p53 protects against Ang II–induced hypertension and endothelial dysfunction. A, Intron–exon structure of the shc1 gene, location of p66shc and p46/52shc promoters, and transcripts of p46/52shc and p66shc. Exons
pifithrin-α (supplemental Figure II). These data suggest that p53 regulates the expression of p66shc in endothelial cells through a transcriptional mechanism and that p53-mediated upregulation of p66shc in endothelial cells has biological relevance. Because of the well-known association of both Ang II and p53 with oxidative stress in vascular cells, we examined the role of p66shc in mediating p53-induced oxidative stress in endothelial cells. Expression of p53 in endothelial cells increased hydrogen peroxide levels, and this increase was abrogated in cells in which p66shc expression was downregulated (Figure 2E), consistent with p66shc being a downstream transcriptional target of p53 in vascular endothelial cells.

The promoter of p66shc is distinct from that of p46/52shc and is located in intron 1 of the human and mouse SHC1 genes.

We searched intron 1 of the human and rodent SHC1 genes for putative p53 binding sites. Nine hundred thirty-eight base pairs upstream of the start codon of human p66shc exists a 24-bp sequence that closely resembles the p53 consensus binding site (Figure 2A), with conservation of key nucleotides important for DNA binding. Similarly, the mouse and rat SHC1 genes possess 22-bp putative p53 binding sites. Using promoter-reporter constructs, we first examined the effect of p53 on p66shc promoter activity. Increasing p53 expression in HEK 293 cells increased the activity of a p66shc promoter fragment that includes the putative p53 binding site (Figure 2C). To determine the importance of this site in p53-stimulated promoter activity, the activity of a truncated p66shc promoter fragment that does not include this site (Figure 2B) was measured. Compared with the full-length promoter, the truncated promoter displayed significantly lower basal and p53-stimulated activity (Figure 2C). To further examine the importance of this putative p53 binding site in p63-stimulated promoter activity, we also constructed a full-length promoter that is mutated at 2 residues within the site (Figure 2B). Two invariant nucleotides that are important for binding to p53 within the p53 consensus binding site were mutated. Basal and p53-stimulated activity of this promoter mutated at critical p53-binding nucleotides was significantly lower when compared with the wild-type p66shc promoter (Figure 2D). Thus, within intron 1 of the shc1 gene, which encodes for the p66shc transcript is shown (bottom). B, Knockdown of p66shc in p66shcRNAi transgenic animals. P66shcRNAi transgenic and age-matched control adenovirus (AdLacZ), aortic rings expressing p53 in the endothelium had significantly impaired endothelium-dependent vasorelaxation (Figure 4A). Compared with rings treated with a control adenovirus (AdLacZ), aortic rings expressing p53 in the endothelium had significantly impaired endothelium-dependent vasorelaxation (Figure 4B), and reduced bioavailable NO (Figure 4C). However, endothelium-independent vasorelaxation was not affected by endothelial p53 expression (Figure 4D). These findings indicate that p53 expression in the endothelium impairs endothelium-dependent vasorelaxation, in part, by decreasing endothelium-derived NO.

The role of p66shc in age-associated endothelial dysfunction has been established. However, because Ang II upregulates p66shc expression in endothelial cells (Figure 1C), we wondered whether p66shc also participates in endothelial...
dysfunction induced by Ang II. To address this, we created a transgenic mouse expressing a small interfering RNA targeted to p66shc. The short hairpin RNA is complementary to a sequence in the coding region of the unique N-terminal CH2 domain of p66shc, which is not a part of the transcripts of p52/46shc (Figure 5A). Mice transgenic for this small interfering RNA (p66shcRNAi mice) had significant knockdown of p66shc in the aorta when compared with their nontransgenic (wild-type) littermates (Figure 5B). Moreover, in comparison with their wild-type littermates, p66shcRNAi mice showed markedly blunted upregulation of aortic p66shc expression in response to Ang II infusion (Figure 5B). We then compared the effect of Ang II infusion on endothelium-dependent vasorelaxation in the wild-type and p66shcRNAi mice. In contrast to wild-type mice, Ang II did not impair endothelium-dependent vasodilatation in p66shcRNAi mice (compare Figure 5C and 5D). Similarly, Ang II infusion decreased bioavailable vascular NO in wild-type but not in p66shcRNAi mice (compare Figure 5E and 5F). Endothelium-independent vasorelaxation was not impaired in either the wild-type or p66shcRNAi mice (Figure 5G and 5H). In parallel with the effect of p66shc knockdown on Ang II–induced vasomotor function, Ang II–induced increase in systolic blood pressure was also significantly blunted in the p66shcRNAi mice (Figure 5I). Because p53 mediates Ang II–induced upregulation of p66shc in endothelial cells (Figure 1C), we also determined the causative role of p53 in Ang II–induced impairment of vasomotor function. To inhibit p53 function, wild-type mice were injected with pifithrin-α during Ang II infusion. Administration of pifithrin-α mitigated Ang II–induced impairment of endothelium-dependent vasorelaxation (Figure 5J), suggesting a role for endogenous p53 in mediating endothelial dysfunction triggered by Ang II. Thus, knockdown of vascular p66shc, or inhibition of p53 function, confers protection against Ang II–induced endothelial dysfunction.

The U6 RNA polymerase III promoter provides high level of constitutive expression across a variety of cell types. Therefore, the p66shc short hairpin RNA is likely to be expressed in most tissues, resulting in global knockdown of p66shc expression in the transgenic mice. Thus, we determined the specific contribution of the endothelium to the difference in Ang II–induced vasomotor dysfunction between the p66shcRNAi and wild-type mice. Endothelium from aortic rings harvested from Ang II–treated p66shcRNAi and wild-type mice was denuded. In contrast to vessels in which the endothelium was intact, p66shc knockdown in endothelium-denuded aortas did not improve acetylcholine-induced relaxation, nor did it decrease vasoconstriction in response to the smooth muscle cell agonist phenylephrine (Figure 6A and 6B). Moreover, similar to endothelium-intact vessels, there was no difference in sodium nitroprusside elicited vascular relaxation in endothelium-denuded vessels derived from Ang II–treated p66shcRNAi and wild-type mice (Figure 6C). Taken together, these findings suggest that p66shc knockdown in vascular layers other than the endothelium does not alter vasoconstriction and vasorelaxation responses and underscore the primary role of p66shc expressed in the endothelium (and not in the vascular smooth muscle or adventitial layer) to Ang II–induced impairment of vasorelaxation.

**Discussion**

P53 is known to upregulate p66shc, although the molecular mechanism underlying this upregulation was hitherto un-
known. The identification of a p53 response/binding element in the promoter region of p66shc indicates that p66shc is a target gene of p53. It is important to point out that in quest of a p53 response element, we examined sequences outside, yet in proximity to, the limits of the promoter of p66shc (−434 to +101 relative to transcription start site) that was previously reported. The finding that this longer promoter (1141 bp) displayed basal activity higher than the truncated promoter (976 bp) (Figure 2C) points to the importance of more distant 5′ regulatory sequences, including the p53 binding sequence, in the regulation of promoter activity. Furthermore, it is noteworthy that although p53 significantly and reproducibly increased the activity of the promoter, this induction was modest (∼3- to 6-fold). This suggests that, in the context of physiological stimuli such Ang II, the newly identified p53 response element may not act in isolation to upregulate p66shc expression but rather synergistically with other regulatory elements in the promoter. Regardless of other cis and trans factors that may cooperate with p53 in regulating p66shc expression, these findings underscore the vital role of p53 in regulating Ang II–stimulated p66shc expression in endothelial cells.

One of the α subunits of heterotrimeric G proteins, Goq (also termed Gaq11), is coupled to the Ang II type 1 receptor (AT1R), and its activation is involved in signal transduction triggered by Ang II. Goq is expressed in endothelial cells. Interestingly, stimulation of Goqα-coupled signaling in cardiomyocytes upregulates p66shc expression suggesting that Goqα may similarly couple AT1R to p66shc expression in vascular endothelial cells. Equally interesting, certain members of the Goq protein superfamily have been recently shown to regulate p53 expression under nongenotoxic conditions.

One limitation of this study is that knockdown of p66shc was not limited to the endothelium. Therefore, although our findings strongly suggest that, in the context of pressor doses of Ang II, endothelial p66shc is the principal mediator of vasomotor dysfunction, we cannot completely exclude the role of p66shc expressed in the vascular smooth muscle and adventitial layers. This may especially be relevant in the setting of other vasoactive agonists that alter vasomotor function. One example of such vasoactive agents may be insulin and insulin-like growth factors, which are mitogens and chemoattractants for vascular smooth muscle cells and lead to phosphorylation of shcA proteins in such cells.

In conclusion, these data provide evidence for a novel, evolutionarily conserved mechanism for the transcriptional control of p66shc expression by p53 and make the argument that both p53 and p66shc play essential roles in mediating Ang II–induced endothelium-dependent impairment of vasomotor function. This hitherto unknown role of endothelial p53 in regulating vascular tone, which, unlike that of p53 expressed in the medial smooth muscle layer, may promote the early development of atherosclerosis, adds an additional layer of complexity to how p53 impacts on vascular physiology and disease.

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Disclosures
None.

References


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Online Figure I. Angiotensin II increases p53 expression in rat aortic endothelial cells (RAEC). Immunoblot for p53 and β-actin in lysates of RAEC challenged with Ang II (100 nM) for the specified times.

Online Figure II. Inhibition of p53 function suppresses basal p66shc and p53 expression. P53 and p66shc protein expression was quantified HUVEC treated with pifithrin-α for 24 hr. Densitometric values expressed relative to untreated cells. * p< 0.05 compared with untreated cells. (n = 3). Representative immunoblot is shown at bottom.
Online Figure I

Ang II (hr)  0  4  5  6  7

p53

β-actin
Online Figure II

Relative p53 expression
(P66shc/β-actin)

Relative p66shc expression
(P66shc/β-actin)

Pifithrin-α (30 µM) - +

*