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Rapid communication

P66shc regulates endothelial NO production and endothelium-dependent vasorelaxation: implications for age-associated vascular dysfunction

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Abstract

The p66shc adaptor protein mediates age-associated oxidative stress. We examined the role of p66shc in endothelial nitric oxide synthase (eNOS) signaling. Overexpression of p66shc inhibited eNOS-dependent NO production. RNAi-mediated down-regulation of endogenous p66shc led to activation of the proto-oncogene ras, and Akt kinase, with a corresponding increase in phosphorylation of eNOS at S1177 (S1179 on bovine eNOS). In rat aortic rings, down-regulation of p66shc suppressed the vasoconstrictor response to phenyephrine that was abrogated by treatment with the NOS inhibitor L-NAME, and enhanced vasodilation induced by sub-maximal doses of acetylcholine. These findings highlight a pivotal role for p66shc in inhibiting endothelial NO production, and endothelium-dependent vasorelaxation, that may provide important mechanistic information about endothelial dysfunction seen with aging.

Keywords: P66shc; Vascular tone; Endothelium; Nitric oxide; Ras

1. Introduction

P66shc belongs to the shcA family of adaptor proteins, which includes p52shc and p46shc. Shc proteins are modular in 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 (RTK), resulting in activation of the membrane-bound GTPase ras [1]. P66shc is unique among shcA proteins because of its distinct structural and functional features. Functionally, p66shc plays a pivotal role in the regulation of intracellular levels of reactive oxygen species (ROS) [2], a feature that the other two shcA proteins do not share. By virtue of its ability to govern ROS levels, p66shc plays an important role in organismal aging [3], and age-associated vascular dysfunction [4]. 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 ROS regulating function of p66shc [5].

In addition to ROS, the vascular redox state is also a function of endothelial nitric oxide (NO) production. The function of p66shc in governing NO production by endothelial NO synthase (eNOS) is not known. We hypothesized that distinct from its effect on intracellular ROS levels, p66shc also influences endothelial-dependent vasodilation by regulating endothelial NO production.

2. Materials and methods

2.1. Aortic ring preparation, ex vivo adenoviral infection, and vascular tension recordings

The procedure for adenoviral infection, and vascular tension recording of rat aortic rings has been previously described

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in [6]. Briefly, male Wistar-Kyoto rats, 12–16 weeks of age, were euthanized by an overdose of Na pentobarbital. Thoracic aortas were harvested, cleaned, and infected ex vivo with the appropriate adenovirus. After 24 hours, the vessels were placed in organ chambers superfused with oxygenated Krebs buffer solution at 37 °C, pH 7.4. All rings were stretched to 3000 mg. One dose of KCl (60 mM) was administered to verify vascular smooth muscle viability. Contractile responses to phenyephrine (PE), and acetylcholine (ACh) were measured and recorded using a myograph system connected to a three-dimensional micromanipulator and a force transducer. Animal protocols were approved by IACUC.

2.2. Cell culture, transient transfections, and recombinant adenoviruses

The COS7 cell line and human umbilical vein endothelial cells (HUVEC) (Clonetics) were used. COS7 cells were transfected with the indicated cDNAs using Lipofectamine 2000 (InVitrogen), as previously described in [6]. The recombinant adenovirus Adp66shcRNAi encodes a short hairpin loop RNA with a 19-mer sequence corresponding to bases 45–63 of the cDNA of p66shc. These nucleotides are in the 330 bp coding region of the N-terminal CH2 domain, and are unique to mRNA of p66shc. Adp66shc was generated by the AdEasy system as described previously in [7]. Adβgal, encoding the inert LacZ gene, was used as a control.

2.3. NO measurements

The NO metabolites nitrite and nitrate were measured using a kit as per manufacturer's recommendations (CalBiochem).

2.4. Ras activity assay and Western blotting

Active (GTP-bound) ras was measured using an active ras pull-down assay kit (UBI). Western blotting was performed using 50 μg protein and standard SDS-PAGE, transfer, and immunoblotting procedures. Antibodies were purchased commercially: shcA (Santa Cruz), Akt, p-Akt, eNOS, and p-eNOS (Cell signaling).

2.5. Statistics

Values shown are mean ± S.E.M. Student's *t*-test for unpaired values was used for statistical analysis. Blots representative of two or three independent experiments are shown.

3. Results and discussion

To determine the role of p66shc in endothelial NO production, we first examined whether over-expression of p66shc affects eNOS-derived NO levels. Expression of exogenous p66shc inhibited eNOS-derived NO, as measured by accumulation of the NO metabolites nitrite and nitrate (Fig. 1A).

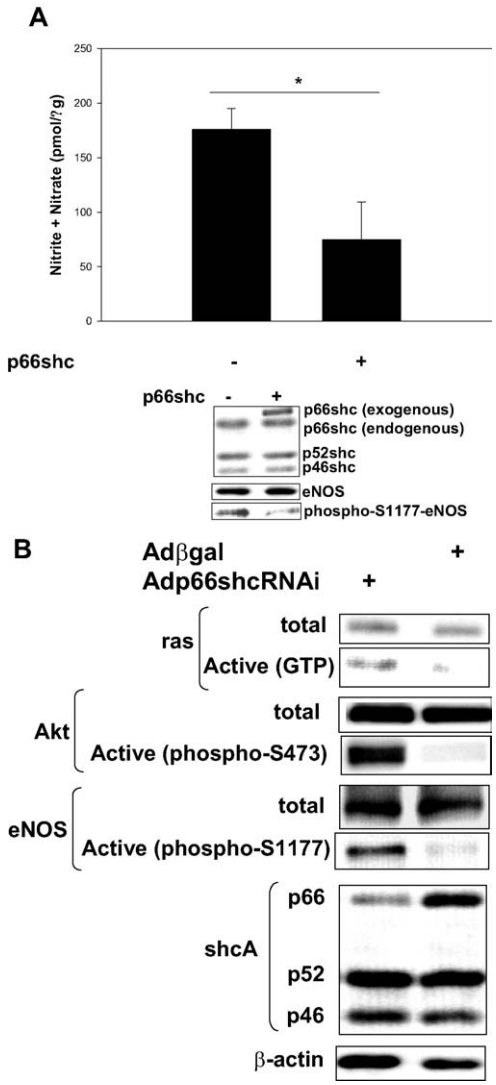

Fig. 1. P66shc inhibits the Ras-PI3K-Akt-eNOS signaling pathway, and suppresses eNOS-dependent NO generation. A) Nitrite + nitrate levels in conditioned media, and phosphorylation of eNOS at S1177 in lysates, of COS7 cells transfected with eNOS ± p66shc. Nitrite + nitrate were measured 48 hours after transfection. Values were obtained by subtracting nitrite + nitrate in cells not transfected with eNOS, and normalized for protein content. * $P < 0.05$. Bottom: Immunoblots showing expression of endogenous shcA proteins, exogenous tagged p66shc, exogenous eNOS, and S1179 phosphorylation of eNOS. B) HUVECs were infected at 500 moi with either Adp66shcRNAi or Adβgal. Forty-eight hours later whole cell lysates were probed for the indicated proteins and phosphoproteins, and subjected to the active ras pull-down assay.

In addition, overexpression of p66shc suppressed basal phosphorylation of eNOS at serine 1179, implying that a reduction in phosphorylation at this residue may be an important mechanism by which p66shc decreases eNOS-derived NO.

We then examined the role of endogenous p66shc in mechanisms that regulate eNOS activity. One such mechanism is the Ras-PI-3K-Akt signaling pathway that phosphorylates human eNOS on serine 1177, and stimulates its sensitivity to calcium [8]. To manipulate endogenous p66shc expression, we constructed an adenovirus, Adp66shcRNAi, with a small hairpin RNA encoding a 19-mer sequence in the