Canonical Wnt Signaling Induces Vascular Endothelial Dysfunction via p66Shc-Regulated Reactive Oxygen Species

Ajit Vikram, Young-Rae Kim, Santosh Kumar, Asma Naqvi, Timothy A. Hoffman, Ajay Kumar, Francis J. Miller Jr, Cuk-Seong Kim, Kaikobad Irani

Objective—Reactive oxygen species regulate canonical Wnt signaling. However, the role of the redox regulatory protein p66Shc in the canonical Wnt pathway is not known. We investigated whether p66Shc is essential for canonical Wnt signaling in the endothelium and determined whether the canonical Wnt pathway induces vascular endothelial dysfunction via p66Shc-mediated oxidative stress.

Approach and Results—The canonical Wnt ligand Wnt3a induced phosphorylation (activation) of p66Shc in endothelial cells. Wnt3a-stimulated dephosphorylation of β-catenin, and β-catenin–dependent transcription, was inhibited by knockdown of p66Shc. Exogenous H2O2-induced β-catenin dephosphorylation was also mediated by p66Shc. Moreover, p66Shc overexpression dephosphorylated β-catenin and increased β-catenin–dependent transcription, independent of Wnt3a ligand. P66Shc-induced β-catenin dephosphorylation was inhibited by antioxidants N-acetyl cysteine and catalase. Wnt3a upregulated endothelial NADPH oxidase-4, and β-catenin dephosphorylation was suppressed by knocking down NADPH oxidase-4 and by antioxidants. Wnt3a increased H2O2 levels in endothelial cells and impaired endothelium-dependent vasorelaxation in mouse aortas, both of which were rescued by p66Shc knockdown. P66Shc knockdown also inhibited adhesion of monocytes to Wnt3a-stimulated endothelial cells. Furthermore, constitutively active β-catenin expression in the endothelium increased vascular reactive oxygen species and impaired endothelium-dependent vasorelaxation. In vivo, high-fat diet feeding–induced endothelial dysfunction in mice was associated with increased endothelial Wnt3a, dephosphorylated β-catenin, and phosphorylated p66Shc. High-fat diet–induced dephosphorylation of endothelial β-catenin was diminished in mice in which p66Shc was knocked down.

Conclusions—p66Shc plays a vital part in canonical Wnt signaling in the endothelium and mediates Wnt3a-stimulated endothelial oxidative stress and dysfunction (Arterioscler Thromb Vasc Biol. 2014;34:2301-2309.)

Key Words: endothelial cells ■ oxidation–reduction ■ p66Shc protein ■ reactive oxygen species ■ Wnt signaling pathway

Wnt/β-catenin (canonical) signaling is an evolutionarily conserved pathway, which plays an important physiological role in proliferation, differentiation, and cell fate speciation.1-3 Canonical Wnt signaling leads to dephosphorylation and stabilization of β-catenin, which then associates with T-cell factor/lymphoid-enhancer binding factor family of transcription factors and regulates expression of Wnt target genes.2 Wnt/β-catenin signaling is involved in multitude of cellular responses in different organ systems, and therefore deregulation of signaling is associated with a host of diseases and syndromes, ranging from schizophrenia to cancer to osteoporosis.2

Animal1 and human5,6 studies show an association between deregulation of Wnt/β-catenin signaling and vascular diseases. Moreover, many Wnt ligands and signaling components are expressed in vascular endothelial cells.7,8 It is also noteworthy that Wnt/β-catenin signaling is augmented in models of aging9 and is upregulated in aged human arteries.10 In addition, the reactive oxygen species (ROS) H2O2 promotes stabilization of β-catenin,11 suggesting a link between oxidative stress and canonical Wnt signaling in aged tissues.

P66Shc belongs to the shcA family of adaptor proteins, which share a common (Src homology-2) SH2 domain, a collagen homology-1 region and phosphotyrosine binding domain.12 P66Shc also possesses a unique amino-terminal collagen homology-2 domain. Phosphorylation of serine 36 in the collagen homology-2 domain occurs in response to variety of stimuli, including UV rays, H2O2 treatment, and growth factor receptor activation. p66Shc, on activation further increases intracellular ROS by promoting its generation and inhibiting the expression of antioxidant enzymes,13 thus, it acts as a...
sensor as well as amplifier of oxidative stress. Genetic ablation studies suggest an important role for p66shc in the regulation of fat accumulation,14 endothelial dysfunction,15–17 and atherosclerosis.20–22

Both canonical Wnt signaling and p66shc have been linked to vascular pathology, but crosstalk between the two remains unknown. In the present study, we tested the hypothesis that Wnt/β-catenin signaling mediates endothelial dysfunction through p66shc.

Materials and Methods

Material and Methods are available in the online-only Supplement. Briefly, vascular reactivity studies were performed in isolated aortas as previously described.21 H2O2 produced by cells was measured in conditioned medium using the Amplex Red probe, as previously described.24

Results

To study canonical Wnt signaling in the endothelium, we first verified its existence in endothelial cells using Wnt3a, the prototypical ligand for activation of the canonical pathway. Recombinant Wnt3a stimulated β-catenin dephosphorylation and expression in bovine aortic endothelial cells (Figure 1A). Conditioned medium containing Wnt3a also stimulated total and dephosphorylated (active) β-catenin in bovine aortic endothelial cells (Figure I in the online-only Data Supplement). In human umbilical vein endothelial cells as well, Wnt3a-conditioned medium and recombinant Wnt3a stimulated dephosphorylation and accumulation of β-catenin in a dose- and time-dependent manner (Figure 1B; Figure IAI to IIC in the online-only Data Supplement). Wnt3a also stimulated β-catenin–dependent transcriptional response mediated by T-cell factor/leukemia enhancing factor in bovine aortic endothelial cells (Figure 1C). Thus, the canonical Wnt signaling machinery is operative in endothelial cells from different vascular beds.

We then asked whether p66shc is required for canonical Wnt signaling. Knockdown of p66shc inhibited Wnt3a-stimulated dephosphorylation and accumulation of β-catenin in bovine aortic endothelial cells and human umbilical vein endothelial cells (Figure 1A and 1B). Similarly, β-catenin–dependent transcription was dependent on p66shc (Figure 1D). Moreover, overexpression of p66shc increased β-catenin–dependent transcription, independent of Wnt3a ligand (Figure 1E). Thus, p66shc is required for canonical Wnt signaling in endothelial cells and is sufficient to stimulate the canonical Wnt pathway. P66shc plays a central role in regulating the redox status of cells and tissues. Therefore, we questioned whether canonical Wnt signaling is dependent on p66shc-regulated ROS. To answer this, we first determined whether canonical Wnt signaling is associated with an increase in ROS in endothelial cells. Wnt3a led to a significant increase in H2O2 levels in endothelial cells (Figure 2A). Moreover, H2O2 increase by Wnt3a was abrogated by small hairpin RNA–mediated knockdown

Nonstandard Abbreviations and Acronyms

HFD high-fat diet
JNK c-jun N-terminal kinase
Lrp5/6 low-density lipoprotein receptor–related protein-5/6
NOX-4 NADPH oxidase-4
ROS reactive oxygen species

Figure 1. P66shc is required for canonical Wnt signaling in endothelial cells. A, Small interfering RNA (siRNA)–mediated knockdown of p66shc in bovine aortic endothelial cell (BAEC) inhibits Wnt3a-stimulated dephosphorylation and increase of β-catenin. B, Small hairpin RNA (shRNA)–mediated knockdown of p66shc in human umbilical vein endothelial cell suppresses Wnt3a-stimulated increase of β-catenin. C, Wnt3a stimulates β-catenin–mediated transcription in BAEC measured by TOP-Flash (TOP) luciferase reporter plasmid. Mutated FOP-Flash (FOP) reporter was used as a negative control. Lithium chloride (LiCl) was used as a positive control (n=3). D, Wnt3a-stimulated TOP-Flash luciferase activity in human embryonic kidney (HEK) 293 cells is suppressed by knocking down p66shc (n=3). E, Expression of p66shc, but not the redox-deficient p66shc S36A, induces β-catenin–mediated transcription (TOP-Flash luciferase activity) in HEK 293 cells (n=3). All values are shown as means±SEM. ***P<0.001 vs indicated group. Immunoblots are representative of 3 experiments. AdLacZ indicates control adenovirus encoding Escherichia coli LacZ; and Adp66shcRNAi, adenovirus-expressing p66shc shRNA.
of p66Shc (Figure 2A). In addition, suppressing oxidative stress with cell-permeable antioxidants N-acetyl cysteine and polyethylene glycol (PEG)-catalase prevented Wnt3a-induced dephosphorylation of β-catenin (Figure 2B and 2C). We then investigated the involvement of NADPH oxidase-4 (NOX-4), the principal NOX expressed in endothelial cells, in canonical Wnt signaling in endothelial cells. Wnt3a upregulated NOX-4 expression and knockdown of NOX-4 with Ad-small hairpin RNA-NOX-4 abrogated Wnt3a-induced dephosphorylation of β-catenin (Figure 2D). Furthermore, H\(_2\)O\(_2\) alone induced dephosphorylation of β-catenin, an effect that was abrogated by siRNA-mediated knockdown of p66Shc (Figure 2E and 2F). These findings underscore the p66Shc-mediated redox-dependent nature of canonical Wnt signaling in endothelial cells and highlight the role of NOX-4 in this signaling.

Phosphorylation of p66Shc on serine 36 is essential for its pro-oxidative function.\(^{12}\) Because p66Shc mediates H\(_2\)O\(_2\) stimulated by Wnt3a, we inquired whether p66Shc is phosphorylated on serine 36 in response to Wnt3a. Wnt3a-conditioned medium and recombinant Wnt3a induced rapid serine 36 phosphorylation of p66Shc in endothelial cells (Figure 2G; Figure IID in the online-only Data Supplement). Inhibition of Wnt signaling with the extracellular Wnt ligand antagonist dickkopf-1 suppressed Wnt3a-stimulated phosphorylation of p66shc (Figure 2G). Moreover, nonphosphorylatable p66\(^{Shc\ A}\) (S36A), which is incapable of promoting oxidative stress, did not increase β-catenin–dependent transcription (Figure 1E). In addition, dephosphorylation of β-catenin induced by p66\(^{Shc\ A}\) was blunted by the antioxidant N-acetyl cysteine (Figure 2H). Taken together, these findings further support a role for p66Shc-mediated redox mechanisms in canonical Wnt signaling.

Because several kinases are known to induce phosphorylation of p66Shc on serine 36, we sought to identify the kinase responsible for Wnt3a-induced p66\(^{Shc\ A}\) phosphorylation. Human umbilical vein endothelial cells were preincubated with specific kinase inhibitors and the effect of Wnt3a on Ser36 phosphorylation of p66\(^{Shc\ A}\) was examined. The c-jun N-terminal kinase (JNK) inhibitor SP600125 inhibited Ser36 phosphorylation of p66\(^{Shc\ A}\), whereas inhibition of mitogen-activated protein kinase kinase with PD98059 or p38MAPK with SB203580 did not (Figure 3A). Moreover, inhibition of JNK, but not p38MAPK or mitogen-activated protein kinase kinase, decreased active and total β-catenin (Figure 3B–3D). These results show that JNK is the principal kinase responsible for
phosphorylation of p66Shc and dephosphorylation of β-catenin in endothelial cells in response to Wnt3a.

To explore the physiological relevance of Wnt signaling to vascular function, we first determined whether canonical Wnt signaling impairs endothelium-dependent vasorelaxation. Incubation of mouse aortas with Wnt3a led to a significant decrease in acetylcholine-stimulated endothelium-dependent vasorelaxation and nitric oxide bioavailability, without affecting sodium nitroprusside-stimulated endothelium-independent vasorelaxation (Figure 4A and 4C; Figure IIIA and IIIB in the online-only Data Supplement). This impairment of endothelium-dependent vasorelaxation was rescued when aortas were preincubated with the Wnt ligand antagonist dickkopf-1 (Figure 4A). We also investigated whether endothelial dysfunction induced by canonical Wnt signaling is mediated by p66Shc. Wnt3a-induced decrease in endothelium-dependent vasorelaxation and NO bioavailability was rescued by small hairpin RNA–mediated knockdown of p66Shc in mouse aortas (Figure 4B and 4C). We further determined the effect of Wnt signaling on vascular function, independent of Wnt ligand. An adenovirus was used to express nonphosphorylatable active β-catenin (S37A) in the endothelium (Figure 5A), thus constitutively activating Wnt signaling independent of Wnt ligand. Expression of β-catenin (S37A) resulted in impairment of endothelium-dependent vasorelaxation (Figure 5B), and a decrease in vascular NO bioavailability (Figure 5C), but did not affect endothelium-independent vasorelaxation (Figure IIIC in the online-only Data Supplement). β-catenin (S37A) expression also increased ROS, both in the endothelial cells (Figure 5D) and in the whole vessel (Figure 5E and 5F). These findings suggest that ROS, in addition to leading to β-catenin dephosphorylation, are also upregulated downstream of

Figure 3. c-jun N-terminal kinase (JNK) phosphorylates p66Shc in response to Wnt3a. A, Wnt3a-induced serine 36 phosphorylation of p66Shc in human umbilical vein endothelial cell (HUVEC) is inhibited by the JNK inhibitor SP600125, but not by the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059, or the p38MAPK inhibitor SB203580. B to D, Wnt3a-induced dephosphorylation and increase of β-catenin in HUVEC is inhibited by the JNK inhibitor SP600125, but not by the MEK inhibitor PD98059, or the p38MAPK inhibitor SB203580. Enzyme-linked immunosorbent assay of active (dephosphorylated) β-catenin/β-actin and total β-catenin/β-actin is shown (n=3). E, Wnt3a induces expression of tumor necrosis factor-α (TNFα) in HUVEC (n=3). F, Constitutively active nonphosphorylatable form of β-catenin (β-catenin (S37A)) upregulates TNFα in HUVECs (n=3). All values are shown as means±SEM. **P<0.001, vs indicated group. Immunoblots are representative of 3 experiments. Adj-β-catenin (S37A) indicates adenovirus-expressing (β-catenin (S37A)); IB, immunoblot; IP, immunoprecipitate; PD, PD98059; SB, SB203580; SP, SP600125; and WCL, whole cell lysate.

Figure 4. Canonical Wnt signaling impairs endothelium-dependent vascular relaxation via p66Shc. A, Wnt3a decreases acetylcholine-induced endothelium-dependent vasorelaxation of mouse aorta, which is reversed by dickkopf-1 (Dkk1-1; n=4–12 aortic rings from 3 mice). Small hairpin RNA–mediated knockdown of p66Shc with Adp66ShcRNAi in mouse aortas rescues Wnt3a-induced decrease in (B) endothelium-dependent vasorelaxation and (C) bioavailable NO (n=5–9 aortic rings from 3 mice). AdLacZ was used as control. All values are shown as means±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs control, #P<0.05, ##P<0.01, and ###P<0.001 vs Wnt3a. AdLacZ indicates control adenovirus encoding Escherichia coli LacZ.
β-catenin. To seek out a potential mechanism for β-catenin–induced ROS, we examined the expression of tumor necrosis factor-α, a target gene of canonical Wnt signaling in other cell types and experimental models,25–27 and one which is well-known to promote endothelial dysfunction.28 Wnt3a, as well as active β-catenin (S37A), led to expression of tumor necrosis factor-α in endothelial cells (Figure 3E and 3F). Taken together, these data show that both ligand-dependent and ligand-independent canonical Wnt signaling impair endothelium-dependent vasorelaxation, the former via p66Shc.

Impairment of endothelium-dependent vasorelaxation is just 1 manifestation of endothelial dysfunction. We measured an additional readout of endothelial dysfunction: adhesion of leukocytes. Wnt3a increased adhesion of U937 monocytic cells to endothelial cells, which was suppressed by knocking down p66Shc (Figure 6A and 6B) and by the antioxidant PEG-catalase (Figure 6C and 6D). However, Wnt3a-induced increase of monocyte adhesion was not affected by inhibition of nitric oxide synthase with N-nitro-l-arginine methyl ester (Figure 6E and 6F). These data show that canonical Wnt signaling also contributes to the inflammatory milieu of a dysfunctional endothelium, leading to adhesion of leukocytes.

To explore the potential role of the Wnt-p66Shc axis in an in vivo model of endothelial dysfunction, we chose a high-fat diet (HFD) feeding–stimulated mouse model of hypercholesterolemia in which p66Shc is known to contribute to vascular oxidative stress.22 HFD feeding of wild-type mice for 16 weeks impaired endothelium-dependent vasorelaxation and NO bioavailability (Figure IV in the online-only Data Supplement) and increased vascular and endothelial Wnt3a expression (Figure 7A and 7B). A similar increase in vascular Wnt3a was observed in apolipoprotein E–deficient mice on a HFD (Figure 7A). HFD feeding also stimulated dephosphorylation of β-catenin (Figure 7C) and expression of c-myc, a target gene of β-catenin–mediated transcription (Figure 7D), in the endothelium. This signifies activation of the canonical Wnt pathway in the endothelium with HFD feeding. Moreover, HFD feeding stimulated serine 36 phosphorylation of p66Shc in the endothelium as well as the media (Figure 7C). To determine the contribution of p66Shc to canonical Wnt signaling in the
intact vasculature, we also subjected mice expressing a p66Shc small hairpin RNA transgene (p66ShcRNAi) to HFD feeding. In these mice, which are protected from vascular fatty streak formation (Figure V in the online-only Data Supplement), dephosphorylation of β-catenin, as well as phosphorylation of p66Shc, was significantly decreased (Figure 7C), illustrating an important role for p66Shc in HFD-stimulated canonical Wnt signaling in the vasculature.

Discussion

Emerging evidence suggests that both oxidative and nitrosative stress play a part in Wnt signaling. NOX-1 modulates Wnt signaling in progenitor cells of the colon29; NOX-1–mediated ROS lead to dissociation of the Wnt signaling mediator (disheveled [Dvl]) from nucleoredoxin, thus promoting Wnt signaling30; nitrosative stress is important in Wnt activation in vascular inflammation. Specific Wnt ligands promote vascular smooth muscle cell proliferation and are upregulated in the neointima of injured vessels.35 In addition, activity of the Wnt coreceptors Fizzled-4 and low-density lipoprotein receptor–related protein-5 (Lrp5) is upregulated in neovascularization associated with oxygen-induced proliferative retinopathy,36 and Wnt signaling is associated with oxidative stress in retinal pigment epithelium31 and diabetic retinopathy.32 Given the role of specific NOXs in endothelial dysfunction,33 it is not surprising that NOX-4 is involved in canonical Wnt signaling in endothelial cells. However, in certain tissues, oxidative stress has also been shown to inhibit canonical Wnt signaling. Pro-osteogenic Wnt signaling in bone is suppressed by oxidative stress.34 Thus, the role of ROS in promoting or inhibiting Wnt signaling may be cell and tissue specific.

Although the role of Wnt signaling in endothelium-dependent vasorelaxation has not been examined previously, Wnt signaling has been associated with vasculopathies and vascular inflammation. Specific Wnt ligands promote vascular smooth muscle cell proliferation and are upregulated in the neointima of injured vessels.35 In addition, activity of the Wnt coreceptors Fizzled-4 and low-density lipoprotein receptor–related protein-5 (Lrp5) is upregulated in neovascularization associated with oxygen-induced proliferative retinopathy,36 and activation of the canonical Wnt pathway in monocytes with Wnt3a leads to monocyte adhesion to endothelial cells via Wnt3a–conditioned media (Wnt3a-CM)–stimulated adhesion of monocytes to HUVEC, which is rescued by small interfering RNA (siRNA)–mediated knockdown of p66Shc. Dephosphorylation of endothelial β-catenin and serine 36 phosphorylation of p66Shc, was significantly decreased (Figure 6), illustrating a role for p66Shc in HFD-stimulated canonical Wnt signaling in the vasculature.
signaling may also play a part in the pathogenesis of pulmonary hypertension, as there is exaggerated expression of β-catenin target genes platelet-derived growth factor receptor and axin in smooth muscle cell overgrowth of pulmonary arterial hypertension lesions. In contrast to a role for Wnt signaling in promoting vascular disease, there is equal evidence that, in the proper context, it is vital for vascular homeostasis. A loss of function mutation in human Lrp6 is associated with early coronary artery disease, and Lrp6 knockout mice on an apolipoprotein E–deficient background develop accelerated atherosclerosis in response to HFD. In addition, dikkopf-1, a Wnt inhibitor, is upregulated in atherosclerosis and promotes inflammatory interaction between platelets and endothelial cells. These contrasting effects of Wnt signaling on the vasculature may be partly explained by the fact that although promoting vascular smooth muscle cell proliferation and endothelial dysfunction through direct effects, Wnt signaling is nevertheless important for systemic glucose and lipid regulation.

Our data demonstrate that JNK is principally responsible for Wnt3a-stimulated p66Shc phosphorylation. JNK participates in both canonical and noncanonical Wnt signaling, and studies suggest that activation of noncanonical signaling antagonizes canonical Wnt signaling. Wnt3a is generally considered a typical ligand for canonical Wnt signaling but it has been shown to activate noncanonical signaling as well. Moreover, Wnt3a can activate JNK and JNK mediates nuclear localization of β-catenin. Thus, there is significant crosstalk between canonical and noncanonical signaling and some promiscuity of the mediators responsible for these 2 forms of Wnt signaling. Although we did not directly examine other readouts of noncanonical signaling, given that JNK mediates Wnt3a-stimulated p66Shc phosphorylation, and is also involved in noncanonical signaling, it would not be surprising if p66Shc plays some part in the noncanonical Wnt pathway in the endothelium.

HFD feeding activates the tumor suppressor p53, and p53 impairs endothelium-dependent vasorelaxation. In addition, endothelial p66Shc is transcriptionally upregulated by p53. Although our data do not shed light on the role of p53 in hypercholesterolemia-stimulated Wnt signaling in the vasculature, it is noteworthy that p53 is upregulated by Wnt/β-catenin signaling and retards Wnt3a-stimulated proliferation and differentiation of mesenchymal progenitor cells. P53 also feeds back to suppress Wnt signaling through microRNA-34–induced downregulation of genes of the Wnt pathway, suggesting that its role, if any, in canonical Wnt signaling in the endothelium may be complex.

ROS have myriad effects on cellular phenotypes. Our data suggest that in endothelial cells, ROS are important upstream mediators but may also act as downstream effectors of canonical Wnt signaling. This dual role of ROS may be mutually re-enforcing, with p66Shc at the center of this relationship (Figure VI in the online-only Data Supplement). Suppression of β-catenin expression and β-catenin–mediated transcription by antioxidants indicates an essential role for ROS in upstream transduction of canonical Wnt signaling in endothelial cells. In addition, ROS induced by active β-catenin in endothelial cells and the vasculature, independent of Wnt ligand, suggest that ROS also function as downstream effectors of endothelial dysfunction triggered by canonical Wnt signaling. In this regard, endothelial tumor necrosis factor-α upregulated by β-catenin–mediated transcription may be an effector that leads to endothelial dysfunction in a paracrine manner. Other Wnt target genes associated with endothelial dysfunction, such as endothelin-1, may be similarly regulated, and function in a similar manner, in the vasculature. The role of p66Shc in transducing ROS-induced Wnt signaling invokes the additional possibility that external oxidative stresses could impair endothelial function via p66Shc-mediated activation of the canonical Wnt pathway.

In conclusion, our findings provide evidence for a novel and direct effect of canonical Wnt signaling on endothelial function and identify p66Shc as an important player in endothelial dysfunction induced by canonical Wnt signaling. In addition, concomitant activation of canonical Wnt signaling and phosphorylation of p66Shc in the endothelium with HFD, together with the requirement of p66Shc in HFD-stimulated endothelial β-catenin dephosphorylation, suggests that the Wnt-p66Shc axis is important in vascular oxidative stress and endothelial dysfunction of hypercholesterolemia.

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Disclosures

None.

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Interaction between Wnt signaling and TNF-α


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Components of the Wnt signaling pathway are expressed in endothelial cells, but the physiological role of this pathway in endothelium-dependent vascular function is not known. This work demonstrates that the canonical Wnt pathway directly impairs endothelium-dependent vasorelaxation by engaging the p66$^{Shc}$ oxidative stress protein. In addition, this work shows upregulation of the canonical Wnt3a ligand, and activation of the canonical pathway, in the vasculature of hypercholesterolemic mice, suggesting that Wnt-stimulated, p66$^{Shc}$-regulated oxidative stress plays a part in endothelial dysfunction associated with hypercholesterolemia. These findings identify disturbance of vascular endothelial homeostasis as a new functional niche for canonical Wnt signaling, adding to its pleiotropic effects.
Canonical Wnt Signaling Induces Vascular Endothelial Dysfunction via $\text{p66}^\text{Shc}$-Regulated Reactive Oxygen Species

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Materials and Methods

Chemicals

Recombinant mouse Wnt3a, and Dkk1 were purchased from R&D Systems. Phenylephrine (PE), acetylcholine (Ach) and L-NAME were procured from Sigma. Antibodies were purchased from InVitrogen, Santa Cruz Biotech, Transduction Labs, Upstate Biotechnology Inc., Abcam, or Cell Signaling.

Cell culture and transfection

HUVECs were purchased from Clonetics (San Diego, CA USA) and cultured in endothelial growth medium (EGM-2, Lonza, Walkersville, MD USA) and were used until passage 10. Human embryonic kidney 293 (HEK 293) cells, L cells, and L-Wnt3a cells were purchased from American Type Culture Collection. Conditioned media was collected from L and L-Wnt3a cells. Bovine aortic endothelial cells (BAEC) were purchased from Cell Applications (San Diego, CA USA). HEK293 cells and BAECs were cultured in DMEM (Mediatech, Inc, Manassas, VA USA) supplemented with 10% FBS and 100 μg/ml streptomycin, and 100 μg/ml penicillin. Cells were transfected with plasmids, validated siRNA-p66Shc or negative control siRNA using Lipofectamine 2000 (InVitrogen, Carlsbad, CA USA) per the recommendations of the manufacturer. Cells were pre-treated with Dkk1 (100 ng/ml) for 3 hrs. pcDNA3.1 (Invitrogen) was used as negative control for the transfections.

Animals and diet

p66Shc siRNA transgenic and their control WT littermate mice on a C57Bl/6 background have been previously described1. The HFD is an adjusted calorie diet that provides 42% calories from fat (TD.88137, Harlan). Mice were placed on this diet for 16 weeks, enough to induce endothelial dysfunction. Animals were sacrificed with CO2 inhalation and aortas rapidly harvested. All the animals were provided access to food and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee and were carried out according to NIH guidelines.

Kinase inhibitors

HUVECs were pre-incubated for 30 min with specific kinase inhibitors: JNK inhibitor SP600125 at 20 μM; MEK inhib then stimulated with Wnt3a for 1 hr. Phosphorylation of p66Shc, total β-catenin and active (dephosphorylated) β-catenin levels were examined by standard immunoblotting.
**Endothelial cell-monocyte adhesion assay**

Adhesion of U937 monocytes to endothelial cells was determined as previously described\(^2\). Briefly, confluent HUVECs were activated by pre-incubating with Wnt3a (100 ng/ml, 1 hr), U937 cells were re-suspended in EGM-2 media and \(2 \times 10^6\) cells were added to each well. U937 cells were incubated with HUVECs for 30 min. and then washed with RPMI-1640 5 times. L-NAME or PEG-catalase was added to HUVECs 1 hr prior to the addition of Wnt3a. Adhesion of U937 to HUVECs monolayer was examined under inverted light microscope (20X). Number of adherent U937 cells per field (20× magnification) was determined and results represent mean of at least three independent experiments.

**Aortic ring preparation and vascular tension recordings**

Thoracic aortas of mice kept on either HFD or ND were used in the study. The animals were rapidly euthanized by CO\(_2\) inhalation. The aorta was carefully dissected, rapidly removed, and placed in ice-cold oxygenated Krebs-Ringer bicarbonate solution. The vessel was carefully cleared of loose connective tissue and cut into 5-10 1.5 mm rings. Aortic rings from each mouse was suspended between two wire stirrups (150 µm) in a 12.5-ml organ chambers of a four-chamber myograph system (DMT Instruments) in 5 ml Krebs-Ringer (95% O\(_2\)-5% CO\(_2\), pH 7.4, 37°C). One stirrup was connected to a three-dimensional micromanipulator, and the other to a force transducer. The mechanical force signal was amplified, digitalized, and recorded (PowerLab 8/30). All concentration-effect curves were performed on arterial rings beginning at their optimum resting tone. This was determined by stretching arterial rings at 10 min intervals in increments of 100 mg to reach optimal tone (≈500 mg). One dose of KCl (60 mM) was administered to verify vascular smooth muscle viability. Cumulative dose-response curve for phenylephrine (PE) (\(10^{-9}-10^{-5}\) M) was obtained by administering the drug in log doses. Endothelium-dependent and –independent vasodilatation was determined by generating dose-response curves to acetylcholine (ACH \(10^{-9}-10^{-5}\) M) and sodium nitroprusside (SNP \(10^{-9}-10^{-5}\) M), respectively on PE (\(10^{-6}\) M) induced pre-contracted vessel. Vasorelaxation evoked by ACh and SNP was expressed as percent relaxation, determined by calculating percentage of inhibition to the pre-constricted tension. NO bioavailability was measured physiologically by determining increase in the contractile response to NOS inhibition (L-NAME \(10^{-4}\) M) in rings pre-constricted with PE (\(10^{-6}\) M). Aortic rings were pre-incubated with recombinant Wnt3a (100 ng/ml), with or without Dkk1 (100 ng/ml), for 24 h.

**Oil-Red-O (ORO) staining**
ORO staining was performed as previously described\(^3\) with some modifications. Briefly, a working solution was prepared by diluting ORO stock (3% ORO in 2-propanol) 6:4 with distilled water and filtering through 0.2-\(\mu\)m filter. Adventitious aortic fat tissue was carefully removed, and aortas were rinsed with 70% 2-propanol for 10 seconds and stained for 30 minutes with ORO working solution, rinsed again with 70% 2-propanol for 10 seconds and then placed in distilled water. Images of stained aorta were captured and individual aortas were kept in 100 \(\mu\)l chloroform/methanol (2:1, v/v) in a 96 well plate on an orbital shaker until the stain was dissolved (5 min.). The absorbance was measured at 490 nM (Wallac 1420 Victor3 Microplate Reader, PerkinElmer, Massachusetts, USA) and dry weight of individual aortic arch was determined. The relative absorbance per mg of dry weight of aortic arch was calculated and data were expressed as % of ND.

**Ex vivo adenoviral infections**

Endothelium-specific gene transfer was achieved ex vivo by incubating freshly isolated aortas from mice, sutured at one end, with 3.0× 10\(^8\) pfu of the appropriate adenoviral stock, and incubated at 37\(^\circ\)C for 4 hr. as previously described\(^4\). The virus was removed and the aorta was then incubated for 24 hrs before sectioning into rings. The viruses used have been previously described\(^5,6\).

**Luciferase reporter assays**

The TOP-Flash (and control FOP-Flash) luciferase reporter plasmids were a kind gift from Randall Moon. TOP-Flash measures β-catenin-mediated TCF/LEF transcriptional activity. The reporter was co-transfected with a renilla luciferase plasmid driven by a constitutive promoter reporter into cells. Firefly and renilla luciferase luminescence were measured using the Dual Luciferase reporter kit (Promega) as per manufacturer’s recommendations. The firefly/renilla ratio was calculated, to normalize for variations in transfection efficiencies.

**Immunoprecipitation, immunoblotting and immunohistochemistry**

Immunoprecipitations were carried out by incubating 2 \(\mu\)g of antibody with 1 mg of cell lysate overnight, followed by 40 \(\mu\)l of protein A- sepharose slurry (Amersham) for 2 h. After washing, immunoprecipitates were boiled in SDS-PAGE gel 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 \(\mu\)g of whole cell lysates was similarly performed. Chemiluminescent signal was developed using Super Signal West Femto substrate (Pierce), blots imaged with a Gel Doc 2000 Chemi...
Doc system (BioRad), and bands quantified using Quantity One software (BioRad). For immunohistochemical studies aortic sections were deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM). These sections were probed with appropriate primary antibodies (Wnt3a, pS36-p66\textsuperscript{shc}, active-β-catenin, β-catenin, c-myc). Polyvalent biotinylated secondary antibody and streptavidin peroxidase (STV–HRP) system was used to amplify the signals, followed by detection with diaminobenzidine as a chromogen. Slides were counterstained with hematoxylin, dehydrated with alcohol and xylene and mounted in DPX.

**ROS levels in HUVEC**

Diffusible H\textsubscript{2}O\textsubscript{2} produced by cells was measured in conditioned medium using the Amplex Red probe, as previously described\textsuperscript{7}.

**In situ quantification of ROS in aortas**

Aortas from wild-type mouse were isolated and infected with adenovirus encoding for either LacZ or activated non-phosphorylatable form of β-catenin (24 hrs), embedded in OCT, sectioned (6 μm), and mounted on glass slides. The sections were rinsed in PBS, and incubated in 10 μM dihydroethidium (DHE) (37°C, 30 minutes; Invitrogen). Slides were mounted, and photographed. Red channel was selected using Photoshop software from at least 5 images of each group and intensity was quantified using ImageJ software.

**Statistical analysis**

Statistical analysis was performed using SPSS (Version 17.0) statistical software. Significance of difference between two groups was evaluated using t-test. For multiple comparisons, one way ANOVA was used and post-hoc analysis was performed with Tukey's test. Results were expressed as Mean ± SEM and considered significant if \( P \) values were ≤ 0.05. All shown data is representative of at least three independent experiments.


Supplemental Figure Legends

Supplemental Fig I. Wnt3a conditioned medium (Wnt3a-CM) increases total as well as active (dephosphorylated) β-catenin in BAEC. Data is representative of 3 experiments.

Supplemental Fig II. (a) Wnt3a-CM increases active β-catenin in HUVEC. (b & c) Recombinant Wnt3a increases β-catenin in HUVEC in a dose and time-dependent manner. (d) Wnt3a (200 ng/ml) stimulates rapid phosphorylation of p66shc in HUVEC. Data is representative of 3 experiments.

Supplemental Fig III. Wnt3a or non-phosphorylatable active β-catenin (S37A) do not inhibit sodium-nitroprusside (SNP)-stimulated endothelium-independent vasorelaxation of mouse aortas. There was no significant difference between the groups. n = 3-5. (a) Ref # Fig. 4a (b) Ref # Fig. 4c (c) Ref # Fig. 4e. All values are shown as Mean ± SEM.

Supplemental Fig IV. High-fat diet (HFD)-feeding leads to (a) impaired endothelium-dependent vasorelaxation, and (b) decreased NO bioavailability. (c) HFD-feeding has no effect on SNP-stimulated endothelium-independent vasorelaxation ND = normal diet. All values are shown as Mean ± SEM. * P < 0.05, *** P < 0.001 vs. ND. N=3-12.

Supplemental Fig V. Decreased HFD-induced aortic fatty streak formation (Oil-Red-O staining) in mice transgenic for p66Shc shRNA (p66ShcRNAi). N = 3-4. All values are shown as Mean ± SEM. * P < 0.05 vs. WT-ND.

Supplemental Fig VI. Schematic showing proposed model of canonical Wnt signaling in the endothelium and vasculature, and the role of p66shc and ROS in this model. LRP: low-density lipoprotein receptor-related protein; Fz: Frizzled receptor. In addition to TNFα, other target genes of β-catenin in the endothelium that could potentially contribute to vascular oxidative stress and endothelial dysfunction are not shown.
Figure I

Active β-catenin

Total β-catenin

β-Actin
Figure IV
Figure V

- WT-ND
- WT-HFD
- p66ShcRNAi-HFD

ORO Absorbance/mg (% of WT-ND)

* indicates significant difference.