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Canonical Wnt signaling induces vascular endothelial dysfunction via p66^{Shc}-regulated reactive oxygen species

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Abstract

Objective—Reactive oxygen species regulate canonical Wnt signaling. However, the role of the redox regulatory protein p66^{Shc} in the canonical Wnt pathway is not known. We investigated if p66^{Shc} is essential for canonical Wnt signaling in the endothelium and determined if the canonical Wnt pathway induces vascular endothelial dysfunction via p66^{Shc}-mediated oxidative stress.

Approach and results—The canonical Wnt ligand Wnt3a induced phosphorylation (activation) of p66^{Shc} in endothelial cells. Wnt3a-stimulated dephosphorylation of β-catenin, and β-catenin-dependent transcription, was inhibited by knockdown of p66^{Shc}. Exogenous hydrogen peroxide (H_2O_2)-induced β-catenin dephosphorylation was also mediated by p66^{Shc}. Moreover, p66^{Shc} overexpression dephosphorylated β-catenin, and increased β-catenin-dependent transcription, independent of Wnt3a ligand. P66^{Shc}-induced β-catenin dephosphorylation was inhibited by antioxidants N-acetyl cysteine and catalase. Wnt3a upregulated endothelial NADPH Oxidase-4 (NOX-4), and β-catenin dephosphorylation was suppressed by knocking down NOX-4 and by antioxidants. Wnt3a increased H_2O_2 levels in endothelial cells and impaired endothelium-dependent vasorelaxation in mouse aortas, both of which were rescued by p66^{Shc} knockdown. P66^{Shc} knockdown also inhibited adhesion of monocytes to Wnt3a-stimulated endothelial cells. Further, 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 p66^{Shc}. High-fat diet-induced dephosphorylation of endothelial β-catenin was diminished in mice in which p66^{Shc} was knocked down.

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Conflicts of interest

None declared.

Conclusion—p66^{Shc} plays a vital part in canonical Wnt signaling in the endothelium, and mediates Wnt3a-stimulated endothelial oxidative stress and dysfunction

Introduction

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 (TCF)-lymphoid-enhancer binding factor (LEF) 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].

Animal[4] and human[5, 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 aging[9] and is upregulated in aged human arteries[10]. In addition, the reactive oxygen species hydrogen peroxide promotes stabilization of β-catenin[11], suggesting a link between oxidative stress and canonical Wnt signaling in aged tissues.

P66^{Shc} belongs to the shcA family of adaptor proteins which share a common (Src homology-2) SH2 domain, a (collagen homology-1) CH1 region and phosphotyrosine binding domain[12]. P66^{Shc} also possesses a unique amino-terminal CH2 domain. Phosphorylation of serine 36 in the CH2 domain occurs in response to variety of stimuli, including UV rays, H_2O_2 treatment, and growth factor receptor activation. p66^{Shc}, upon 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 p66^{Shc} in the regulation of fat accumulation[14], endothelial dysfunction[15–19], and atherosclerosis[20–22].

Both canonical Wnt signaling and p66^{Shc} 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 p66^{Shc}.

Material and Methods

Material and methods are available in the online-only Supplement. Briefly, vascular reactivity studies were performed in isolated aortas as previously described[23]. H_2O_2 produced by cells was measured in conditioned medium using the Amplex Red probe, as previously described[24].

Results

In order 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 (BAEC) (Fig 1a). Conditioned medium containing Wnt3a also stimulated total and dephosphorylated (active) β-catenin in BAEC (Supplemental Fig I). In human umbilical vein endothelial cells (HUVEC) as well, Wnt3a conditioned medium and recombinant Wnt3a stimulated dephosphorylation and accumulation of β-catenin in a dose- and time-dependent manner (Fig 1b and Supplemental Fig IIa–c). Wnt3a also stimulated β-catenin-dependent transcriptional response mediated by T-cell factor/Leukemia enhancing factor (TCF/LEF) in BAEC (Fig 1c). Thus, the canonical Wnt signaling machinery is operative in endothelial cells from different vascular beds.

We then asked if $p66^{Shc}$ is required for canonical Wnt signaling. Knockdown of $p66^{Shc}$ inhibited Wnt3a-stimulated dephosphorylation and accumulation of β-catenin in BAEC and HUVEC (Fig 1a and 1b). Similarly, β-catenin-dependent transcription was dependent on $p66^{Shc}$ (Fig 1d). Moreover, overexpression of $p66^{Shc}$ increased β-catenin-dependent transcription, independent of Wnt3a ligand (Fig 1e). Thus, $p66^{Shc}$ is required for canonical Wnt signaling in endothelial cells and is sufficient to stimulate the canonical Wnt pathway.

$P66^{Shc}$ plays a central role in regulating the redox status of cells and tissues. Therefore, we questioned if canonical Wnt signaling is dependent on $p66^{Shc}$-regulated reactive oxygen species. To answer this, we first determined if canonical Wnt signaling is associated with an increase in reactive oxygen species in endothelial cells. Wnt3a led to a significant increase in hydrogen peroxide (H_2O_2) levels in endothelial cells (Fig 2a). Moreover, H_2O_2 increase by Wnt3a was abrogated by shRNA-mediated knockdown of $p66^{Shc}$ (Fig 2a). In addition, suppressing oxidative stress with cell-permeable anti-oxidants N-acetyl cysteine (NAC) and PEG-catalase prevented Wnt3a-induced dephosphorylation of β-catenin (Fig 2b & c). 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-shRNA-NOX-4 abrogated Wnt3a-induced dephosphorylation of β-catenin (Fig. 2d). Further, H_2O_2 alone induced dephosphorylation of β-catenin, an effect which was abrogated by siRNA-mediated knockdown of $p66^{Shc}$ (Fig. 2e & f). These findings underscore the $p66^{Shc}$-mediated redox-dependent nature of canonical Wnt signaling in endothelial cells, and highlight the role of NOX-4 in this signaling.

Phosphorylation of $p66^{Shc}$ on serine 36 is essential for its pro-oxidative function[12]. Because $p66^{Shc}$ mediates H_2O_2 stimulated by Wnt3a, we inquired if $p66^{Shc}$ is phosphorylated on serine 36 in response to Wnt3a. Wnt3a conditioned medium and recombinant Wnt3a induced rapid serine 36 phosphorylation of $p66^{Shc}$ in endothelial cells (Fig 2g and Supplemental Fig IId). Inhibition of Wnt signaling with the extra-cellular Wnt ligand antagonist Dickkopf-1 (Dkk1) suppressed Wnt3a-stimulated phosphorylation of p66shc (Fig 2g). Moreover, non-phosphorylatable $p66^{Shc}$ (S36A), which is incapable of promoting oxidative stress, did not increase β-catenin-dependent transcription (Fig 1e). In addition, dephosphorylation of β-catenin induced by $p66^{Shc}$ was blunted by the anti-oxidant NAC (Fig 2h). Taken together, these findings further support a role for $p66^{Shc}$-mediated redox mechanisms in canonical Wnt signaling.

Since several kinases are known to induce phosphorylation of $p66^{Shc}$ on serine 36, we sought to identify the kinase responsible for Wnt3a-induced $p66^{Shc}$ phosphorylation. HUVEC were pre-incubated with specific kinase inhibitors and the effect of Wnt3a on Ser36 phosphorylation of $p66^{Shc}$ was examined. The c-jun N-terminal kinase (JNK) inhibitor SP600125 inhibited Ser36 phosphorylation of $p66^{Shc}$, while inhibition of mitogen-activated kinase kinase (MEK) with PD98059, or p38MAPK with SB203580 did not (Fig. 3a). Moreover, inhibition of JNK, but not p38MAPK or MEK, decreased active and total β-catenin (Fig. 3b–d). These results show that JNK is the principal kinase responsible for phosphorylation of $p66^{Shc}$ 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 if 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 (Fig 4a & c, Supplemental Fig IIIa–b). This impairment of endothelium-dependent vasorelaxation was rescued when aortas were pre-incubated with the Wnt ligand antagonist Dkk1 (Fig 4a). We also investigated if endothelial dysfunction induced by canonical Wnt signaling is mediated by $p66^{Shc}$. Wnt3a-induced decrease in endothelium-dependent vasorelaxation and NO bioavailability was rescued by shRNA-mediated knockdown of $p66^{Shc}$ in mouse aortas (Fig 4b & c). We further determined the effect of Wnt signaling on vascular function, independent of Wnt ligand. An adenovirus was used to express non-phosphorylatable active β-catenin (S37A) in the endothelium (Fig 5a), thus constitutively activating Wnt signaling independent of Wnt ligand. Expression of β-catenin (S37A) resulted in impairment of endothelium-dependent vasorelaxation (Fig 5b), and a decrease in vascular NO bioavailability (Fig 5c), but did not affect endothelium-independent vasorelaxation (Supplemental Fig IIIc). β-catenin (S37A) expression also increased ROS, both in endothelial cells (Fig 5d), and in the whole vessel (Fig 5e & f). These findings suggest that ROS, in addition to leading to β-catenin dephosphorylation, are also upregulated downstream of β-catenin. To seek out a potential mechanism for β-catenin-induced ROS, we examined the expression of tumor necrosis factor-α (TNFα), 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 TNFα in endothelial cells (Fig. 3e & f). Taken together, these data show that both ligand-dependent and ligand-independent canonical Wnt signaling impairs endothelium-dependent vasorelaxation, the former via $p66^{Shc}$.

Impairment of endothelium-dependent vasorelaxation is just one 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 $p66^{Shc}$ (Fig 6a & b) and by the antioxidant PEG-catalase (Fig 6c & d). However, Wnt3a-induced increase of monocyte adhesion was not affected by inhibition of nitric oxide synthase with L-NAME (Fig 6e & f). 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-p66^{Shc} axis in an in vivo model of endothelial dysfunction, we chose a high-fat diet feeding-stimulated mouse model of hypercholesterolemia in which p66^{Shc} is known to contribute to vascular oxidative stress[22]. High-fat diet feeding of wild-type mice for 16 weeks impaired endothelium-dependent vasorelaxation and NO bioavailability (Supplemental Fig IV), and increased vascular and endothelial Wnt3a expression (Fig 7a & b). A similar increase in vascular Wnt3a was observed in ApoE$^{-/-}$ mice on a high-fat diet (Fig 7a). High-fat diet feeding also stimulated dephosphorylation of β-catenin (Fig 7c) and expression of c-myc, a target gene of β-catenin-mediated transcription (Fig 7d), in the endothelium. This signifies activation of the canonical Wnt pathway in the endothelium with high-fat diet feeding. Moreover, high-fat diet feeding stimulated serine 36 phosphorylation of p66^{Shc} in the endothelium as well as the media (Fig 7c). To determine the contribution of p66^{Shc} to canonical Wnt signaling in the intact vasculature, we also subjected mice expressing a p66^{Shc} shRNA transgene (p66^{Shc}RNAi) to high-fat diet feeding. In these mice, which are protected from vascular fatty streak formation (Supplemental Fig V), dephosphorylation of β-catenin, as well as phosphorylation of p66^{Shc}, was significantly decreased (Fig 7c), illustrating an important role for p66^{Shc} in high-fat diet-stimulated canonical Wnt signaling in the vasculature.

Discussion

Emerging evidence suggests that both oxidative and nitrosative stress plays a part in Wnt signaling. NADPH oxidase 1 (NOX1) modulates Wnt signaling in progenitor cells of the colon[29]; NOX1-mediated ROS lead to dissociation of the Wnt signaling mediator Dvl from nucleoredoxin, thus promoting Wnt signaling[30]; nitrosative stress is important in Wnt activation in diabetic nephropathy[31]; and Wnt signaling is associated with oxidative stress in retinal pigment epithelium[27], 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 previously examined, Wnt signaling has been associated with vasculopathies and vascular inflammation. Specific Wnt ligands promote vascular smooth muscle cell proliferation and are upregulated in the neo-intima of injured vessels[35]. In addition, activity of the Wnt co-receptors Fizzled4 and 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 but decreased trans-endothelial migration[37]. Furthermore, Wnt signaling may also play a part in the pathogenesis of pulmonary hypertension, as there is exaggerated expression of β-catenin target genes PDGF receptor and axin in smooth muscle cell overgrowth of pulmonary arterial hypertension lesions[38]. 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[5, 6], and Lrp6 knockout mice on an ApoE$^{-/-}$ background develop accelerated

atherosclerosis in response to high-fat diet[4]. In addition, Dkk1, a Wnt inhibitor, is upregulated in atherosclerosis and promotes inflammatory interaction between platelets and endothelial cells[39]. These contrasting effects of Wnt signaling on the vasculature may be partly explained by the fact that, though promoting vascular smooth muscle cell proliferation and endothelial dysfunction through direct effects, Wnt signaling is nevertheless important for systemic glucose and lipid regulation[5, 40].

Our data demonstrate that JNK is principally responsible for Wnt3a-stimulated $p66^{Shc}$ phosphorylation. JNK participates in both canonical and non-canonical Wnt signaling[41, 42], and studies suggest that activation of non-canonical signaling antagonizes canonical Wnt signaling[43, 44]. Wnt3a is generally considered a typical ligand for canonical Wnt signaling but it has been shown to activate non-canonical signaling as well[45, 46]. Moreover, Wnt3a can activate JNK and JNK mediates nuclear localization of β-catenin[42]. Thus, there is significant crosstalk between canonical and non-canonical signaling and some promiscuity of the mediators responsible for these two forms of Wnt signaling. Although we did not directly examine other readouts of non-canonical signaling, given that JNK mediates Wnt3a-stimulated $p66^{Shc}$ phosphorylation, and is also involved in non-canonical signaling, it would not be surprising if $p66^{Shc}$ plays some part in the non-canonical Wnt pathway in the endothelium.

High-fat diet-feeding activates the tumor suppressor p53[47], and p53 impairs endothelium-dependent vasorelaxation[15, 48]. In addition, endothelial $p66^{Shc}$ is transcriptionally upregulated by p53[15]. 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[49]. P53 also feeds back to suppress Wnt signaling through microRNA-34-induced downregulation of genes of the Wnt pathway[50], 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 $p66^{Shc}$ at the center of this relationship (Supplemental Fig. VI). 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, suggests that ROS also function as downstream effectors of endothelial dysfunction triggered by canonical Wnt signaling. In this regard, endothelial TNFα upregulated through β-catenin-mediated transcription may be one effector which leads to endothelial dysfunction in a paracrine manner. Other Wnt target genes associated with endothelial dysfunction, such as endothelin-1[51], may be similarly regulated, and function in a similar manner, in the vasculature. The role of $p66^{Shc}$ in transducing ROS-induced Wnt signaling invokes the additional possibility that external oxidative stresses could impair endothelial function via $p66^{Shc}$-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 $p66^{Shc}$ as an important player in endothelial dysfunction induced by canonical Wnt signaling. In addition, concomitant activation of canonical Wnt signaling and phosphorylation of $p66^{Shc}$ in the endothelium with high-fat diet, together with the requirement of $p66^{Shc}$ in high-fat diet-stimulated endothelial β-catenin dephosphorylation, suggests that the Wnt-$p66^{Shc}$ axis is important in vascular oxidative stress and endothelial dysfunction of hypercholesterolemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

Ach	acetylcholine
BAECs	bovine aortic endothelial cells
DKK-1	dickkopf-1
Dvl	disheveled
Fz	Frizzled receptor
HEK	human embryonic kidney
HFD	high-fat diet
HUVECs	human umbilical vein endothelial cells
JNK	c-jun N terminal kinase
L-NAME	N-nitro-L-arginine methyl ester
LRP-5/6	LDL-receptor-related protein-5/6
MEK	mitogen activated protein kinase kinase
NAC	N-acetyl cysteine
ND	normal diet
NO	nitric oxide
PE	phenylephrine

SNP	sodium nitroprusside
TCF/LEF	T -cell factor/lymphoid-enhancer binding factor
Wnt3a-CM	Wnt3a-conditioned media
NOX-4	NADPH oxidase-4

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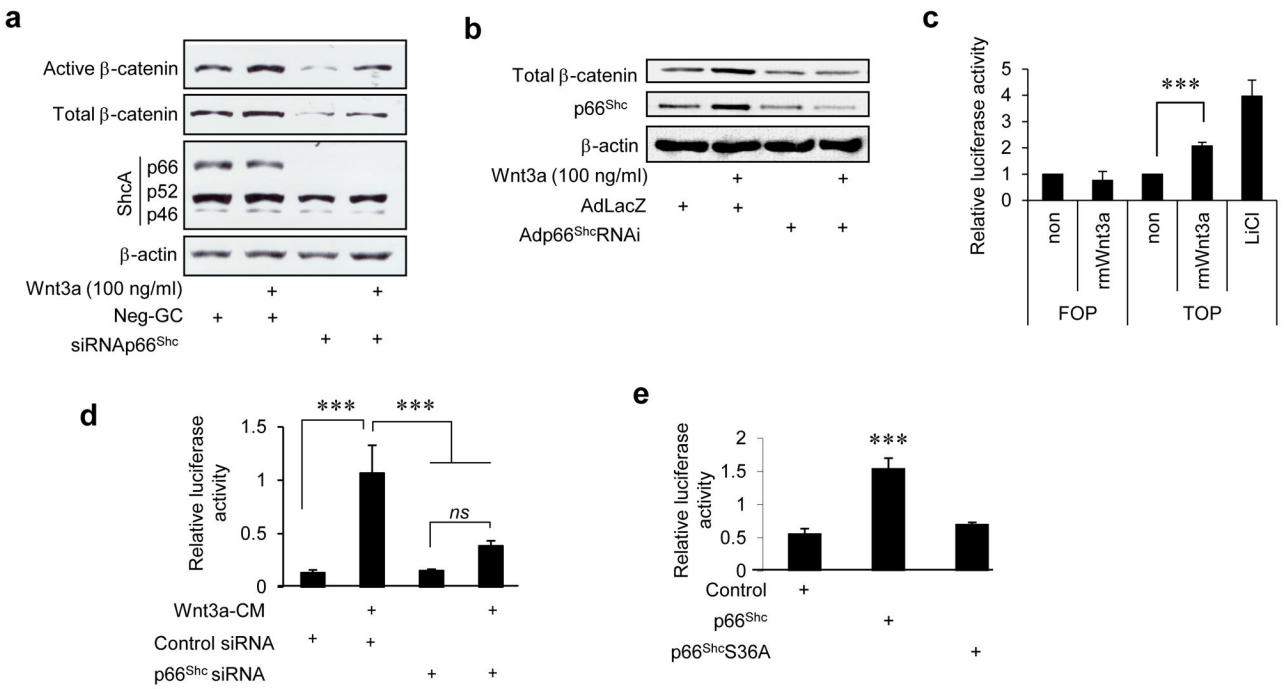

Fig. 1. P66^{Shc} is required for canonical Wnt signaling in endothelial cells

a) siRNA-mediated knockdown of p66^{Shc} in BAEC inhibits Wnt3a-stimulated dephosphorylation and increase of β-catenin. b) shRNA-mediated knockdown of p66^{Shc} in HUVEC suppresses Wnt3a-stimulated increase of β-catenin. Adp66^{shc}RNAi: adenovirus expressing p66^{shc} shRNA; AdlacZ: control adenovirus encoding E. Coli LacZ. 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 HEK 293 cells is suppressed by knocking down p66^{Shc} (n=3). e) Expression of p66^{Shc}, but not the redox deficient p66^{Shc} S36A, induces β-catenin-mediated transcription (TOP-Flash luciferase activity) in HEK 293 cells (n=3). All values are shown as mean ± SEM. *** $P < 0.001$ *vs.* indicated group. ns = not significant. Immunoblots are representative of three experiments.

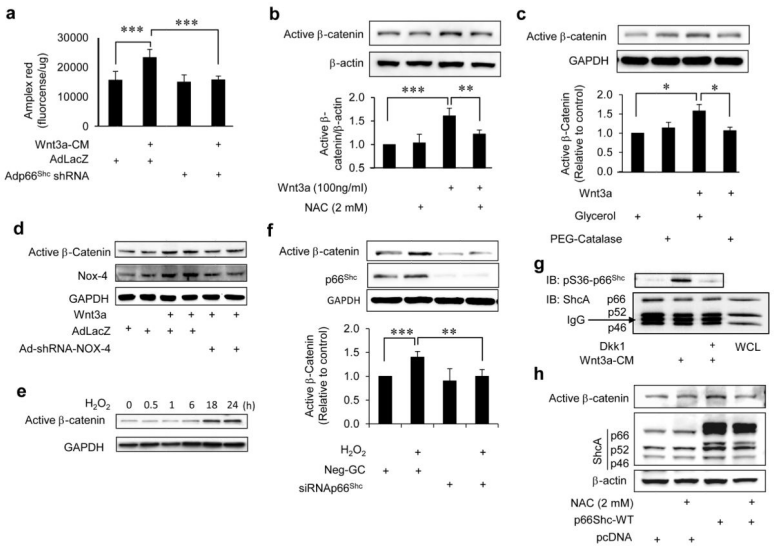

Fig. 2. P66^{Shc}-regulated reactive oxygen species mediate canonical Wnt signaling in endothelial cells

a) Wnt3a conditioned medium (Wnt3a-CM)-induced H_2O_2 in HUVEC is inhibited by shRNA-mediated knockdown of p66^{Shc} (n=3). Adp66^{shc}RNAi: adenovirus expressing p66^{shc} shRNA; AdlacZ: control adenovirus encoding E. Coli LacZ. b) Wnt3a-induced dephosphorylation of β-catenin in HUVEC is suppressed by the anti-oxidant NAC. Densitometry of active β-catenin/β-actin is shown (n=3). c) Wnt3a-induced dephosphorylation of β-catenin in HUVEC is suppressed by the anti-oxidant PEG-Catalase. Densitometry of active β-catenin/GAPDH is shown (n=3). Glycerol (vehicle) was used as control. d) NOX-4 is upregulated by Wnt3a in HUVEC, and Wnt3a-stimulated dephosphorylation of β-catenin is suppressed by shRNA-mediated down-regulation of NOX-4 (n=3). Ad-shRNA-NOX4: adenovirus expressing NOX-4 shRNA. AdLacZ was used as control. e) H_2O_2 dephosphorylates β-catenin in HUVEC. f) siRNA-mediated downregulation of p66^{Shc} suppresses H_2O_2-induced dephosphorylation of β-catenin in HUVEC (n = 4). g) Wnt3a-CM stimulates phosphorylation of p66^{Shc} on serine 36, which is blocked by Dkk1. WCL: whole cell lysate. h) p66^{Shc}-induced dephosphorylation of β-catenin in HUVEC is inhibited by NAC. All data is representative of at least three independent experiments and values are shown as mean ± SEM. ** $P < 0.01$, *** $P < 0.001$ *vs.* indicated group. Immunoblots are representative of 3–4 experiments.

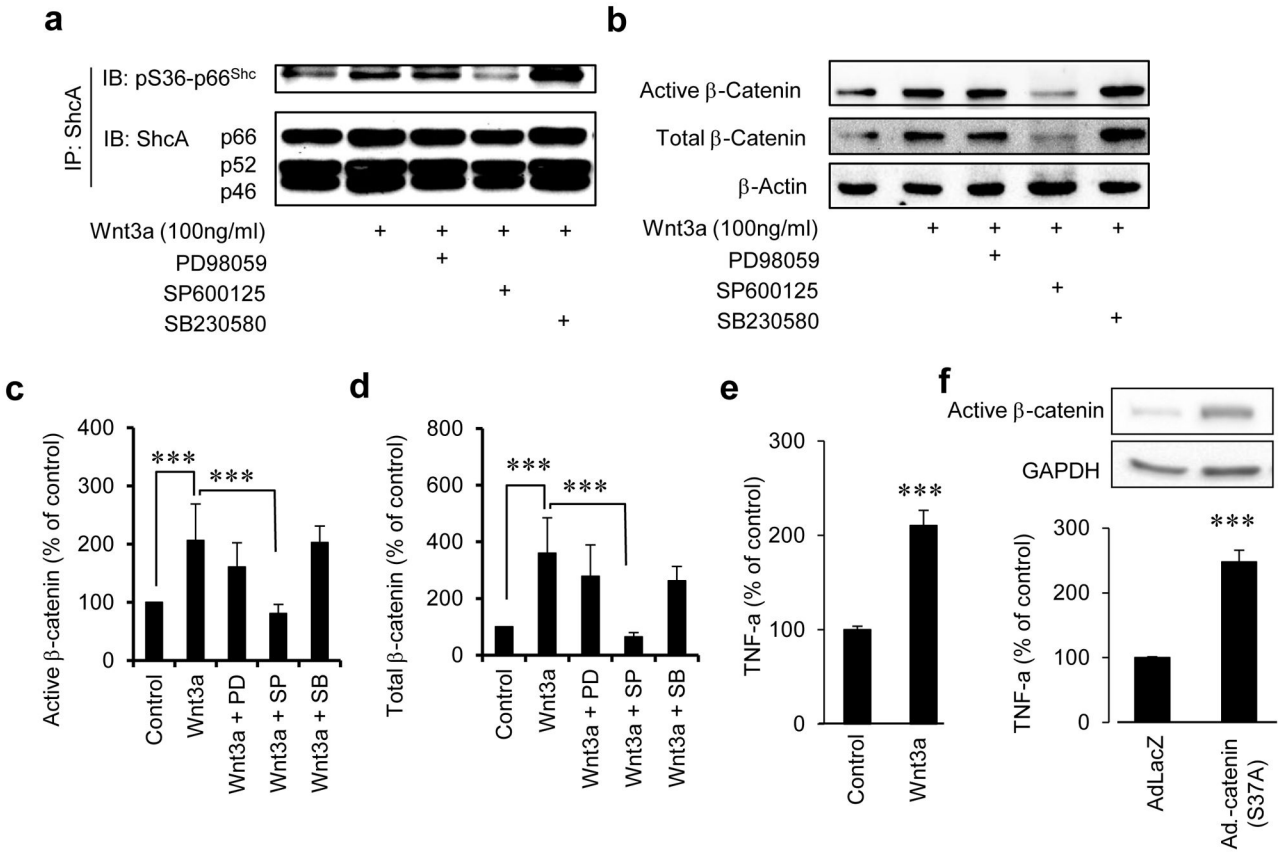

Fig. 3. JNK phosphorylates p66^{Shc} in response to Wnt3a

a) Wnt3a-induced serine 36 phosphorylation of p66^{Shc} in HUVEC is inhibited by the JNK inhibitor SP600125, but not by the MEK inhibitor PD98059, or the p38MAPK inhibitor SB203580. b–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. Densitometry 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 non-phosphorylatable form of β-catenin [β-catenin (S37A)] upregulates TNFα in HUVECs (n =3). Adβ-catenin (S37A): adenovirus expressing β-catenin (S37A). All values are shown as mean ± SEM. *** $P < 0.001$, *vs.* indicated group. WCL; Whole cell lysate, PD; PD98059, SP; SP600125, SB; SB203580. Immunoblots are representative of three experiments.

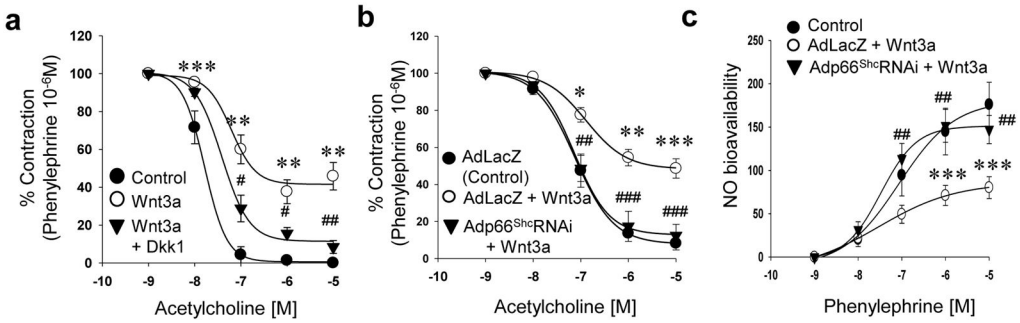

Fig. 4. Canonical Wnt signaling impairs endothelium-dependent vascular relaxation via p66^{Shc}

a) Wnt3a decreases acetylcholine-induced endothelium-dependent vasorelaxation of mouse aortas, which is reversed by Dkk1 (n = 4 – 12 aortic rings from three mice). shRNA-mediated knockdown of p66^{Shc} with Adp66^{Shc}RNAi in mouse aortas rescues Wnt3a-induced decrease in b) endothelium-dependent vasorelaxation, and c) bioavailable NO. (n = 5 – 9 aortic rings from three mice). AdLacZ was used as control. All values are shown as Mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* Control, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ *vs.* Wnt3a.

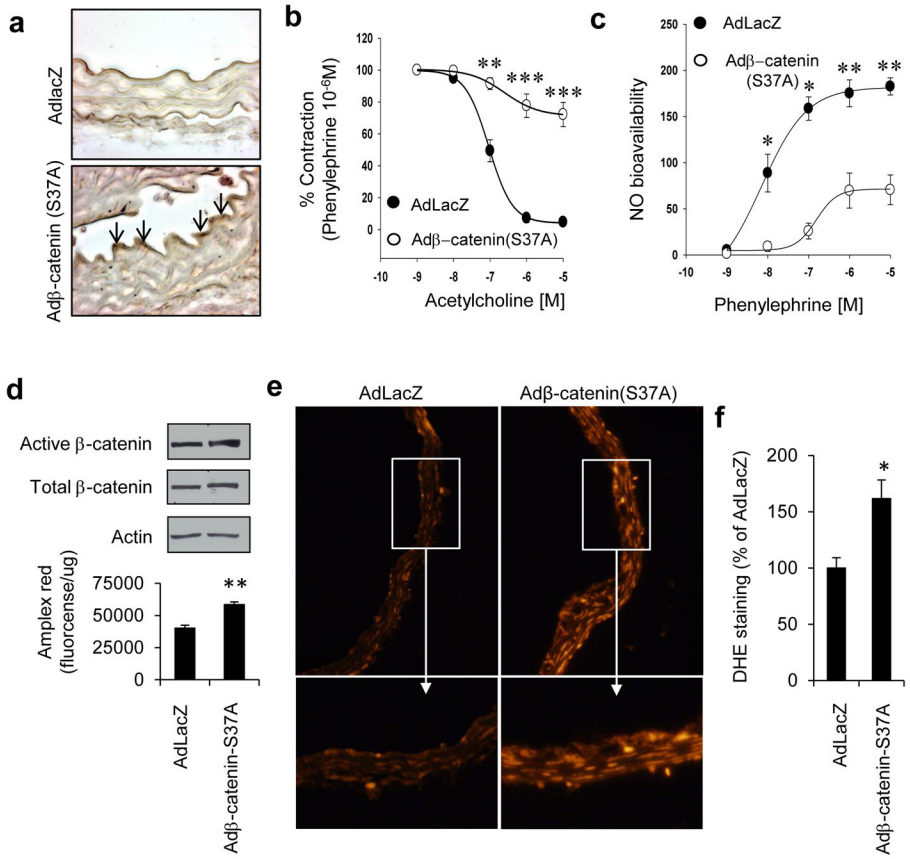

Fig. 5. Active β-catenin induces endothelial and vascular oxidative stress, and impairs endothelium-dependent vasorelaxation

Expression of active (non-phosphorylatable) β-catenin (S37A) in endothelium of mouse aortas (a) decreases b) endothelium-dependent vasorelaxation, and c) NO bioavailability (n = 4 – 12 aortic rings from three mice). AdLacZ was used as control. d) β-catenin (S37A) induces H_2O_2 (Amplex red fluorescence) in HUVEC. e–f) β-catenin (S37A) induces ROS (DHE fluorescence) in mouse aortas. Representative photomicrographs and immunoblots are shown. Adβ-catenin (S37A): adenovirus expressing β-catenin (S37A). All values are shown as Mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* AdLacZ.

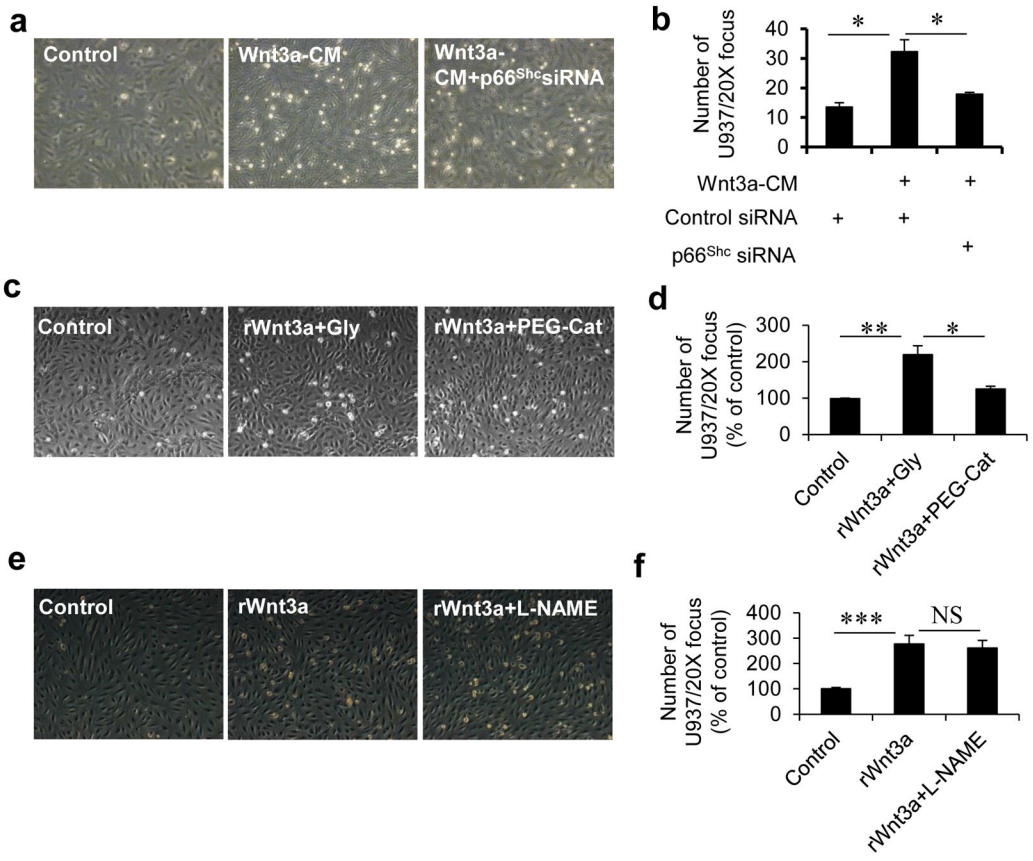

Fig. 6. Canonical Wnt signaling stimulates adhesion of monocytes to endothelial cells via p66^{Shc} and ROS

a) Representative photographs showing Wnt3a-CM-stimulated adhesion of U937 monocytic cells to HUVEC, which is rescued by siRNA-mediated knockdown of p66^{Shc} (n=3). b) Quantification of adherent monocytes in (a). c) Representative photographs showing effect of catalase on Wnt3a-stimulated adhesion of monocytes to HUVEC (n=3). Glycerol vehicle (Gly) was used as control. d) Quantification of adherent monocytes in (c). e) Representative photographs showing effect of NOS inhibitor L-NAME on Wnt3a-stimulated adhesion of monocytes to HUVEC (n=3). f) Quantification of adherent monocytes in (e). All the values are shown as Mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS=not significant. rWnt3a: recombinant Wnt3a.

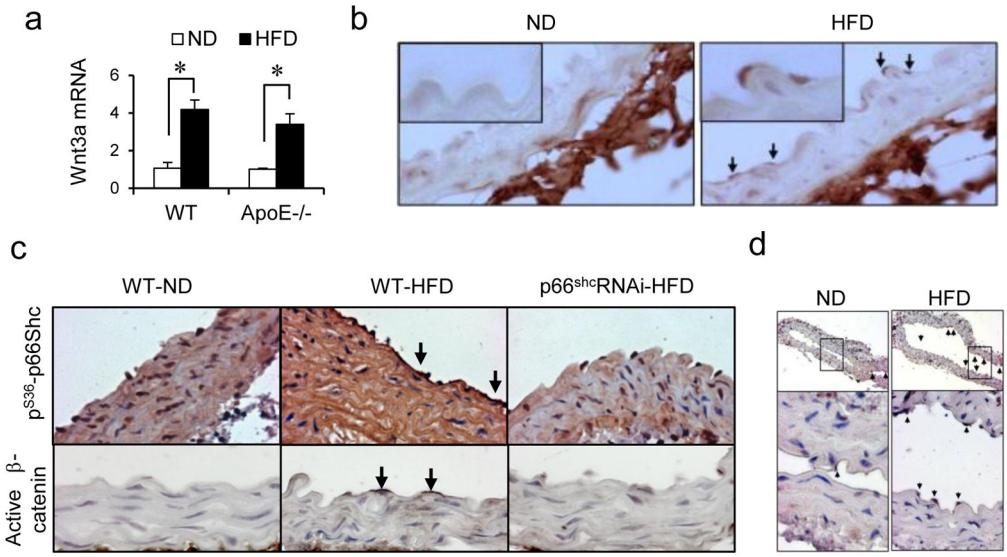

Fig. 7. High-fat diet activates endothelial canonical Wnt signaling via p66^{Shc}

(a) High-fat diet (HFD) feeding increases Wnt3a mRNA in whole aorta of wild-type C57Bl/6 (WT) and ApoE$^{-/-}$ mice (n = 3). (b) Representative photomicrographs showing increased immunostaining for Wnt3a in aortic endothelium of WT HFD-fed mice. ND=normal diet. c) High-fat diet feeding stimulates dephosphorylation of vascular β-catenin and serine 36 phosphorylation of p66^{Shc}. Dephosphorylation of endothelial β-catenin is suppressed in mice transgenic for p66^{Shc} shRNA (p66^{Shc}RNAi). d) High-fat diet feeding increases endothelial expression of β-catenin target gene c-myc in WT mice. All the values are shown as Mean ± SEM. * $P < 0.05$ *vs.* indicated group.