# Endothelial cell autonomous role of Akt1: Regulation of vascular tone and ischemia-induced arteriogenesis

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## Abstract

**Objective:** The importance of PI3K/Akt signaling in the vasculature has been demonstrated in several models, as global loss of Akt1 results in impaired postnatal ischemia- and VEGF-induced angiogenesis. The ubiquitous expression of Akt1, however, raises the possibility of cell-type dependent Akt1-driven actions, thereby necessitating tissue-specific characterization.

**Approach & Results:** Herein, we utilized an inducible, endothelial-specific Akt1-deleted adult mouse model (Akt1iECKO) to characterize the endothelial cell autonomous functions of Akt1 in the vascular system. Endothelial targeted ablation of Akt1 reduces eNOS phosphorylation and promotes both increased vascular contractility in isolated vessels and elevated diastolic blood pressures throughout the diurnal cycle *in vivo*. Furthermore, Akt1iECKO mice subject to the hindlimb ischemia model display impaired blood flow and decreased arteriogenesis.

**Conclusions:** Endothelial Akt1 signaling is necessary for ischemic resolution post-injury and likely reflects the consequence of NO insufficiency critical for vascular repair.

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## INTRODUCTION

Nitric oxide (NO) is an important mediator of cardiovascular homeostasis through its ability to exert physiological effects on numerous cells types of the vasculature. NO coordinates several biological functions, including blood flow, proportional vascular remodeling, and angiogenesis. In the endothelium, NO is primarily generated through endothelial nitric oxide synthase (eNOS) in response to local agonists (*i.e.* bradykinin, acetylcholine) and alterations in hemodynamics. NO can subsequently diffuse through the plasma membrane to the underlying vascular smooth muscle layer to regulate cGMP-dependent vascular relaxation and systemic blood pressure. Enzyme activity is also essential for NO diffusion into the lumen, where binding to hemoglobin in erythrocytes provides circulating NO bioequivalents.

The *in vivo* regulation of eNOS is a complex process that involves multiple interdependent control mechanisms such as lipidation, subcellular localization, intracellular calcium, substrate availability, and phosphorylation at key activation sites, namely serine 1177 (in human eNOS; mouse: S1176; bovine: S1179)[1,2]. Phosphorylation of eNOS at S1177 has previously been shown to occur at low levels of intracellular calcium (*i.e.* with shear stress, insulin)[3,4], highlighting the various avenues of eNOS activation. While several kinases have been identified for eNOS-S1177 phosphorylation (*i.e.* PKA, AMPK)[5], S1177 phosphorylation occurs *in vivo* predominantly through Akt[6]. The protein kinase Akt is a major hub for several signal transduction pathways through its ability to phosphorylate numerous downstream targets directly involved in various cellular processes including proliferation, cell survival, and metabolism – all critical for vascular remodeling[7,8]. Akt has also been shown to preferentially phosphorylate the eNOS isoform over other NOS isoforms, as co-transfection of Akt with nNOS and iNOS does not induce NO release[4]. Biochemical approaches validate S1176 phosphorylation as the main activation site in intact cells, as a 'loss-of-function' mutation to alanine (S1176A) abolishes Akt-dependent eNOS phosphorylation and reduces NO release. Similarly, a 'gain-of-function' mutation to mimic phosphorylation (S1176D) results in significantly enhanced *in vitro* eNOS enzyme activity[3,4]. We have additionally shown the functional importance of S1176 phosphorylation in vasomotor tone regulation, as S1179D adenoviral delivery restores EC-dependent vasodilatory responses in pressurized arteries from eNOS knockout (KO) mice[9]. Furthermore, genetic knock-in approaches indicate that Akt1 is the primary kinase for eNOS *in vivo*, as introduction of the S1176D phospho-mimic mutation in global Akt1 KO mice rescues the profound defects in revascularization after hindlimb ischemia (HLI) and wounding these mice[6].

Of the three Akt isoforms (Akt1/Akt2/Akt3), endothelial cells (EC) express predominantly Akt1, the major isoform involved in regulation of cardiovascular function[10–13]. We have previously shown the isoform-dependent role of Akt in vascular remodeling, as ischemic limb repair is severely impaired in global Akt1 KO mice[10]. Unlike the pronounced defects seen in Akt1 KO mice upon injury, global Akt2 KO mice display normal hindlimb flow recovery, paralleling the *in vitro* observation of preferential eNOS-S1177 phosphorylation through the Akt1 isoform when compared to the Akt2 isoform[10,11]. On a whole organism level, however, global loss of Akt1 results in severe growth defects both *in utero* and postnatal life, as Akt1[KO] mice remain smaller than average throughout adulthood[14,15]. The obvious growth retardation together with the increased propensity to apoptosis in global Akt1 KO mice thus limits the interpretation of how the Akt1 isoform regulates adult vascular remodeling. Furthermore, the protein kinase Akt1 is positioned as a key signaling intermediate in all cell types, thereby confounding theoretical predictions as to the tissue-specific role of Akt1.

Here, we use an inducible, endothelial-specific Akt1-null adult mouse model (Akt1iECKO) to address the EC-specific, post-natal role of Akt1 *in vivo*. Akt1iECKO mice display phenotypes indicative of impaired eNOS function and consequent endothelial dysfunction. We show that endothelial Akt1 ablation promotes a spontaneous hypertensive phenotype associated with EC dysfunction. Furthermore, Akt1iECKO mice subject to HLI display diminished blood flow recovery where microCT imaging indicates an associated impairment of arteriogenesis. Collectively, the cardiovascular phenotypes that arise due to the postnatal deletion of Akt1 in EC supports the critical role of the Akt-eNOS signaling axis in the regulation of vascular function *in vivo*.

## MATERIALS & METHODS

### Inducible endothelial-specific Akt1 conditional mice

The Akt1[flox/flox16] were bred to the Cdh5-Cre-ERT2[17] mice to obtain inducible, endothelial-targeted Akt1 mice, as previously described[11]. Young adult, male mice (~4–5wks of age) were injected with tamoxifen (100ug/g total body weight) via intraperitoneal delivery for 7 consecutive days. Phenotyping assessments were performed 6 weeks post-tamoxifen administration (~10–12 wks of age).

### En face immunostaining

Thoracic aortas were isolated from control and Akt1iECKO immediately after sacrifice. Aortas (or indicated arteries) were isolated and cut longitudinally for *en face* immunostaining. The isolated tissues were incubated at 37°C in serum-free base media for ~4hrs prior to *ex vivo* treatment with Angiopoetin1 (500ng/mL) or PDGF-BB (50 ng/mL) for the indicated times. Samples were then fixed in 4% PFA/PBS and permeabilized overnight in TNB blocking buffer (0.1M Tris pH7.4, 150mM NaCl, 0.2% Triton-X-100, 0.5% blocking reagent) for whole-mount analysis. Aortas were stained with rat anti-VECD (BD555289, 1:200), mouse anti-GM130 (BD610822, 1:100), rabbit anti-panAkt (Cell Sig #9272, 1:200), and rabbit anti-p-eNOS-S1176 (Cell Sig #9571, 1:200). Images were acquired using a Leica SP5 confocal microscope with the Leica Application Suite (LAS)

software. Images reflect a z-stack compression of the endothelial layer using the sequential scan mode with a HCX PL APO lambda blue 63x/1.40 oil objective.

### NO bioavailability (Electric Paramagnetic Resonance)

Electric paramagnetic resonance was applied to measure hemoglobin-bound NO in whole blood, as previously described[18]. In brief, mice were euthanized by $CO_2$ inhalation for subsequent blood isolation from the inferior vena cava. Samples were immediately flash-frozen in liquid $N_2$ for later spectrometer analysis. Mice harboring germline eNOS knock-in mutations (S1176D$^{+/+}$ and S1176A$^{+/+}$)[6] and lacking eNOS [19] were used as gating controls for Hb-NO signal detection. All mice were given standard chow to ensure fixed levels of nitrate/nitrite content to prevent dietary influences on NO bioavailability.

### Wire myography

Wire myography was performed, as previously described[20]. Thoracic aortas were isolated and cleaned under a dissecting scope to remove residual fat and connective tissues. Aortic rings of uniform length were suspended using two tungsten wires through the lumen and subsequently mounted onto myograph chambers containing Krebs buffer solution (95% $O_2$/5% $CO_2$, 37°C). Aortic rings were set to a resting tension of 1.5g under isometric conditions and allowed to equilibrate. Following equilibration, aortic rings were subject to several rounds of KCl contraction followed by contractile-induced dose response curves. For relaxation studies, aortic rings were pre-constricted to ~80% maximal constriction prior to acetylcholine dose-curve generation. Increasing concentrations of the desired agents were administered at half-log increments where real-time responses were recorded using Chart5 (AD Instruments). Control and Akti1ECKO aortic rings were studied simultaneously, isolating at least 3–4 aortic rings per mouse. Tension-response curve data reflect a nonlinear regression curve (log [agonist] vs. response) using Prism 6.0 (GraphPad Software).

### Blood pressure telemetry

Radiotelemetric blood pressure catheters were implanted in the right carotid artery of adult male (10–12 wks old) mice and allowed to recover 1 week from surgery, as previously described[21,22]. Mice were housed in individually controlled environments with a 12-hr light/dark cycle with free access to food and water. Parameters of interest were measured at 1-minute intervals for 7 consecutive days. Data reflect averaged values over a 3-day reading period.

### Hindlimb ischemia

The hindlimb ischemia (HLI) model was performed, as previously described[23,24]. In brief, the left femoral artery and the proximal portion of the saphenous artery is exposed, ligated, and excised. Ischemic and non-ischemic limb perfusion is measured pre-, post-surgery, 1-, 2-, 3-, and 4-weeks after surgery using Laser Doppler scanning (LDI, Moor Instruments Ltd). Perfusion data is analyzed and reported as a ratio of flow in the injured relative to contralateral leg values. S-Nitroco-N-acetyl-DL penicillamine (SNAP) was injected locally (1mg/mL, 30uL injection) where indicated prior to Laser Doppler scanning and flow measurements.

## MicroCT imaging

2D microCT scans were obtained using a GE eXplore MS Micro-CT System, as previously described[23]. Mice were administered 2% adenosine prior to delivery of the contrast agent to dilate the vascular bed and definitively assess the effects on anatomy and vascular remodeling rather than vascular tone. Vessel density analyses was performed on captured images, as previously described[23].

## Statistical Analysis

All data are shown as mean ± standard error of mean (SEM). Statistical significance was determined based on a p-value calculation of $p < 0.05$ where all comparisons reflect a 2-WAY ANOVA, Bonferroni analyses.

# RESULTS

## Inducible deletion of adult endothelial Akt1 expression

Although viable, global Akt1-null mice exhibit significant neonatal mortality and are substantially smaller than WT littermate controls[14,15]. Therefore, we utilized an inducible, tissue-specific approach to bypass potential retardation effects during gestation/growth as a result of global Akt1 loss. Inducible, endothelial targeted (Cdh5-Cre-ERT2) Akt1 mice were generated using Cre-loxP technology, as described previously [11]. Young adult mice (~4–5 weeks of age) were administered tamoxifen for 7 consecutive days via intraperitoneal injection for adult phenotyping at ~10–12weeks of age (Supp. Fig. 1A). While global Akt1 deletion yields significantly smaller mice, inducible deletion does not affect total body weight (Supp. Fig. 1B), thereby eliminating body mass as a variable. *En face* whole-mount immunofluorescent imaging of the endothelial layer from various arterial vessels indicate near complete ablation of total Akt1 protein expression (Supp. Fig. 2), thus validating the inducible deletion strategy. The Akt antibody was determined to be specific, as immunofluorescent labeling is undetectable in the endothelium of global Akt1 KO mice (Supp. Fig. 3).

## Endothelial Akt1 deletion attenuates angiopoietin-1 (Ang1)- and platelet-derived growth factor (PDGF)-stimulated eNOS phosphorylation

Akt1 is the major isoform in the endothelium necessary for mediating pro-angiogenic signaling events[13] and has been demonstrated by several groups as a major kinase for eNOS phosphorylation and subsequent eNOS activation[3,4,10,25]. Therefore, we sought to assess eNOS function using an *ex vivo* approach to validate the endothelial-targeted Akt1 KO mouse model[11]. In brief, segments of the thoracic aorta were isolated from adult Akt1iECKO mice along with respective littermate controls and incubated with Ang1, a prototypical PI3K/Akt agonist to induce eNOS phosphorylation on S1176[26-29]. The phospho-eNOS antibody was determined to be specific for S1176, as immunofluorescence labeling detected phospho-eNOS-S1176 signal in control vessels but not in vessels isolated from mice harboring a 'loss-of-function' mutation in the Akt phosphorylation site (eNOS-S1176A mice[6]; Supp. Fig. 4). As seen in Figure 1A and 1B, e*n face* staining for phospho-eNOS-S1176 demonstrates enhanced perinuclear phospho-eNOS levels[30] in response to

acute Ang1 treatment, where this effect is significantly reduced in Akt1iECKO mice. We similarly stimulated vessels with PDGF-BB, a potent growth factor that activates Akt in the endothelium and induces endothelium-dependent relaxation in intact vessels[31,32]. PDGF-BB treatment of control aortas induces eNOS phosphorylation, thus corroborating previous observations where PDGF-BB treatment produced NO-mediated vessel relaxation[32], as endothelial-specific Akt1 deletion reduces eNOS-S1176 phosphorylation levels (Supp. Fig. 5A, quantified in 5B).

### Endothelial loss of Akt1 decreases NO bioavailability

The transfer of eNOS-derived NO to hemoglobin in circulating erythrocytes relies on the diffusional distance and serves as a critical determinant of plasma NO bioavailability[33]. Thus, the formation of hemoglobin-bound NO (Hb-NO) is directly proportional to the amount of bioavailable NO[34]. Due to the stable nature of Hb-NO under anaerobic conditions, Hb-NO levels measured by electron paramagnetic spin resonance (EPR) techniques in isolated mouse blood[35] reflects eNOS-derived NO levels in blood. Therefore, we measured Hb-NO levels in Akt1iECKO adult mice to determine whether the loss of endothelial Akt1 affected NO bioavailability (Figure 1C). Venous whole blood was collected from adult mice and Hb-NO was measured using EPR, as previously described[18]. Genetically modified eNOS mutant mice either mimicking (S1176D) or lacking (S1176A) eNOS phosphorylation[6] were additionally assessed together with eNOS KO as thresholding controls to determine the window of detection. All mice were given standard chow and food restricted overnight to ensure fixed levels of nitrate/nitrite content to prevent dietary influences on NO bioavailability measurements. Although higher than the 'loss-of-function' eNOS-S1176A mutant, Akt1iECKO mice show significantly less Hb-NO in blood compared to WT littermate controls, highlighting the importance of the Akt1-eNOS axis in regulating NO bioactivity.

### Endothelial loss of Akt1 decreases basal NO levels and impairs vascular function

*Ex vivo* examination of adult Akt1iECKO aortas clearly demonstrate blunted levels of eNOS phosphorylation. To test the significance of these findings in intact vessels, myography was used to examine vascular function in aortic rings from Akt1iECKO mice. Aortic rings isolated from both control and Akt1iECKO adult mice were subjected to various agonists, and tension-response curves were generated. Typical contraction and relaxation responses were observed in control mice, with an EC50 for phenylephrine (PE) and acetylcholine (Ach) matching previously reported values[36] (Figure 2A **and** 2B). However, aortic rings from Akt1iECKO mice had increased sensitivity to the contractile actions of PE and KCl (Figure 2A and 2C), where pre-incubation of vessels with the NOS inhibitor, L-NAME, normalized the PE response in Akt1iECKO mice (Figure 2D). This response suggests that tension-induced release of NO, or basal NO production, was impaired in vessels from Akt1iECKO mice. Ach-dependent vessel relaxation in conduit vessels occurs primarily through the release of endothelial-derived NO[37]. Interestingly, Ach-mediated reductions in vascular tone were virtually indistinguishable between the groups (Figure 2B), demonstrating that endothelial Akt1 is not critical for Ach-mediated NO production, which occurs primarily through $Ca^{2+}$/CaM-dependent mechanisms[2,38].

## Endothelial-specific loss of Akt1 significantly increases diastolic blood pressure

Arterial pressure is of particular importance in maintaining vascular function and response, relying heavily on shear stress-mediated endothelial NO production[39]. The observed decrease in eNOS phosphorylation and circulating Hb-NO together with the increased sensitivity to PE suggests that overall systemic blood pressure may be affected in Akt1iECKO mice. Therefore, arterial blood pressure and heart rate was monitored in intact, conscious mice for over 72 hours using implanted telemetry devices[40]. Akt1iECKO mice display elevated diastolic pressures independent of the time of day (Figures 3A **and** 3B) and an associated inclination toward increased mean arterial pressures (Supp. Fig. 6A). Previous studies using similar telemetry-based blood pressure measurements in global Akt1[KO] mice report non-significant trends toward increased arterial pressure[41], thus denoting the Akt1iECKO phenotype as the first report of hypertension resulting from Akt1 impairment. Other parameters, such as systolic blood pressure, heart rate, and activity levels, were comparable between control and adult Akt1iECKO mice (Figure 3C **and** 3D, Supp. Fig. 6B).

## Endothelial loss of Akt1 results in impaired blood flow recovery and decreased arteriogenesis upon hindlimb ischemia (HLI)

In a model of HLI, the global loss of PI3K$\gamma$[42] or Akt1[10] results in critical limb ischemia and limb loss concomitant with marked impairment in arteriogenesis and angiogenesis. Nevertheless, the observed defects in global Akt1 deficient mice likely reflects a complex response, involving multiple cells types (*e.g.* EC, SMC, macrophages), all of which express the Akt1 isoform. We first assessed baseline arterial density in the EC-inducible Akt1 deletion model using a phase contrast agent together with microCT imaging. Arterial vessels were maximally dilated to definitively assess the vascular network rather than vascular tone, where quantification did not indicate any underlying deficits in artery number prior to hind-limb injury between both groups (data not shown). We subsequently performed the HLI model in Akt1iECKO and littermate controls to assess the role of EC-specific Akt1 expression in this injury response. In brief, the left femoral artery was ligated and removed, upon which blood flow is measured over time using Laser Doppler imaging. Perfusion rates in the surgically-manipulated leg were normalized to the uninjured contralateral limb for blood flow quantification. In control mice, an acute decrease in blood flow was followed by progressive recovery of flow over 28 days post-injury. In contrast, flow recovery in Akt1iECKO was significantly impaired by 14 days and persisted to 28 days post-ischemia when compared to littermate controls (Figure 4A and 4B). To assess arterial density after injury, microCT imaging was conducted 14 days post-HLI[43]. MicroCT analyses indicate a significant decline in the number of smaller arteries both above and below the knee in Akt1iECKO mice post-ischemia (Figure 4C-E), suggesting that collateral remodeling and/or the development of new arterial branches was impaired by the loss of the Akt1 isoform in ECs.

In order to reinforce the importance of Akt1-mediated eNOS activation for vascular recovery, a rescue experiment was performed using S-Nitroso-N-acetyl-DL-penicillamine (SNAP)[44], as an NO donor. Global Akt1 KO mice were used for rescue experiments since the deficit in blood flow after HLI is much larger compared to reduced flow recovery in the

Akt1iECKO mice[10]. As seen in Supp Fig. 7, administration of SNAP with a single-injection downstream of the injury site resulted in near immediate flow recovery in the injured limb of control mice, with even greater flow increases in global Akt1 KO mice. The timely increase in blood flow suggests the presence of a remodeled, yet dysfunctional and constricted vasculature in global Akt1 KO mice, where eNOS activation may be critical for injury resolution through both neovessel formation and vasomotor tone regulation.

## DISCUSSION

Akt1 controls many signaling pathways that regulate cell survival, morphogenesis and growth[7]. While global Akt1-null mice have been instrumental in elucidating the *in vivo* biological role of Akt1, the contribution of Akt1 signaling from a cell-specific perspective has only recently begun to be explored[11,16,45–47]. Here, we show that endothelial loss of Akt1 using an inducible, postnatal EC-specific strategy results in spontaneous hypertension, decreased NO bioavailability, and impaired recovery to tissue limb ischemia – all indicative of impaired NO production. These data are surprising given that eNOS can be phosphorylated on S1176 *in vitro* by several kinases including other AGC kinases (protein kinase A, G, C) and AMPK[48]. The Akt1iECKO phenotypes indicative of impaired NO production suggest that physiologically, eNOS-S1176 phosphorylation *in vivo* through Akt1 is crucial for 'NO tone' and blood pressure control.

A common feature of endothelial dysfunction is diminished NO bioavailability, where eNOS KO mice are an extreme state of NO deficiency.[1] Regardless of the methodology used for blood pressure measurements, eNOS KO mice are consistently hypertensive[19,49–52] and display an associated increase in cardiac mass and function[52]. Akt1 has been well-established as a major kinase for eNOS phosphorylation[3,4,6] and manipulation of Akt activity *in vivo* through use of viral transfection methods in combination with arterial flow measurements indicate that short-term hyperactivation of Akt1 results in augmented NO release, enlarged vessel diameter and enhanced blood flow[53]. Additionally, EC-specific transgenic expression of Akt1 increases NO production and reduces injury-induced neointima formation[54]. While global Akt1 KO mice present phenotypes similar to eNOS KO, such as decreased homozygote offspring number and reduced adult body weight[14,19], global Akt1 KO mice are not hypertensive[11,41]. Normal blood pressures in global Akt1 KO mice suggests that other kinases may regulate eNOS, or that the global loss of Akt1 promotes compensation by other expressed Akt isoforms given the significant overlap in substrate specificity for Akt1 versus Akt2 in the endothelium[11]. Thus, the hypertensive phenotype in adult Akt1iECKO mice emphasizes the importance of the endothelial Akt1-eNOS axis *in vivo*[6] and suggests that adaptive changes likely occur during development that preclude an obvious blood pressure phenotype in global Akt1 KO mice.

Moreover, cardiac output rates derived from echocardiography measurements did not indicate any differences between Akt1iECKO and littermate controls (data not shown). Given the direct relationship between blood pressure and peripheral resistance, the observed pressure elevation together with the lack of change in cardiac output suggests that the Akt1iECKO mice likely exhibit increased systemic vascular resistance. While smaller resistance arteries have been conventionally reliant on endothelium-derived hyperpolarizing

factor (EDHF) as the predominant mediator of vascular tone regulation, the recent discovery of hemoglobin alpha (Hbα) in the myoendothelial junctions of resistance arteries suggest a role for NO scavenging[55]. The localized nature of Hbα at the myoendothelial junction (MEJ) may serve as a chelator of eNOS-derived NO to favor EDHF activity while maintaining control over NO levels. Previous groups have also shown the importance of eNOS expression on peripheral vascular tone, as partial deletion of eNOS increases coronary vascular tone[56] and pulmonary resistance[57], hence complementing the impaired vascular tone seen in isolated vessels from Akt1iECKO mice (Figure 2).

Vascular remodeling occurs in both development and under adult pathological conditions, requiring various adaptations in response to the physiological environment and/or pathological stimuli. Angiogenesis denotes the *de novo* formation of capillaries from pre-existing post-capillary venules and is largely initiated by hypoxic events, whereas arteriogenesis denotes the remodeling of pre-existing collaterals or arterioles/capillaries, which is generally ischemia-independent and driven through hemodynamic and inflammatory factors (*i.e.* shear stress, wall tension)[58,59]. In experimental models of hindlimb ischemia, arteriogenesis is stimulated through a marked increase in fluid shear stress in arterial conduit vessels, providing a driving force to promote collateralization[60]. In the distal lower limb, vascular remodeling is primarily mediated through tissue ischemia, resulting in the upregulation of several growth factors critical for vascularization. eNOS-derived NO serves as a naturally occurring, compensatory mechanism to regulate hemodynamic changes in both normal and ischemic tissue[61]. eNOS function is also essential for native collateral formation, immediate flow recovery post-ligation, and cellular recruitment in the remodeling vasculature – all of which are critical for efficient arteriogenesis[62,63]. The defective flow recovery in adult Akt1iECKO mice herein likely reflects the combinatorial effects of impaired eNOS-derived NO generation given the established role of the Akt1 kinase in eNOS-S1179 phosphorylation[6]. However, the impairment in blood flow and arteriogenesis in Akt1iECKO mice is markedly less than that observed in global Akt1 KO [10] or eNOS KO mice[64], implying that other kinases may contribute to eNOS activation under these conditions or that expression of Akt1 is also critical in SMCs and macrophages for proper ischemic injury repair.

Recent work using conditional EC-specific Akt1 deletion mice has shown that the Akt1 isoform is necessary for developmental retinal angiogenesis through regulation of EC proliferation and survival[11], two well-described functions of Akt and downstream substrate phosphorylation. Furthermore, the global deletion of Akt2 in combination with EC-specific deletion of Akt1 did not further impair the developing retinal vasculature. In contrast, deletion of endothelial Akt1 in adult mice had a mild effect on cardiac arteriogenesis (quantified via microCT), and this effect was markedly exacerbated by the global deletion of Akt2[45], implying functional redundancy of these isoforms in the adult coronary vasculature. Mechanistically, the loss of both isoforms in EC, and Akt2 in smooth muscle, reduce endothelial expression of the Notch ligand, Jagged1, thereby impairing Notch-Jagged regulation of vascular stability. The means by which Akt as a kinase regulates this pathway are not clear, as an Akt substrate has not yet been identified to explain the reduced Jagged1 levels. Prior work has shown that Akt2 exerts a more prominent role in determining vascular SMC phenotype and function[65,66]. Global Akt2 KO mice are additionally more susceptible

to aortic aneurysms[67], further demonstrating a role for the Akt2 isoform in vascular tone regulation and/or vessel compliance through an NO-independent pathway. Nevertheless, our studies indicate that *in vivo* eNOS activity is mediated primarily through the endothelial expression of Akt1 where postnatal deletion results in defects consistent with decreased NO bioavailability.

Collectively, our data supports the importance of the Akt1-eNOS signaling axis in intact endothelium given its primary role in NO production and vasomotor tone regulation. Akt1iECKO mice display phenotypes indicative of NO insufficiency as seen through impaired agonist-induced eNOS phosphorylation, blunted endothelial NO production *in vivo*, diastolic hypertension, and defects in post-ischemic arteriogenesis. Hence, PI3K-Akt1-NO signaling in EC is critical for vascular tone regulation and adaptive vessel remodeling.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## HIGHLIGHTS

- Endothelial-specific Akt1 ablation in adult mice results in spontaneous hypertension

- Adult, inducible, endothelial-targeted Akt1 deletion results in decreased NO bioavailability

- Endothelial loss of Akt1 signaling impairs injury-induced arteriogenesis, indicative of impaired eNOS function
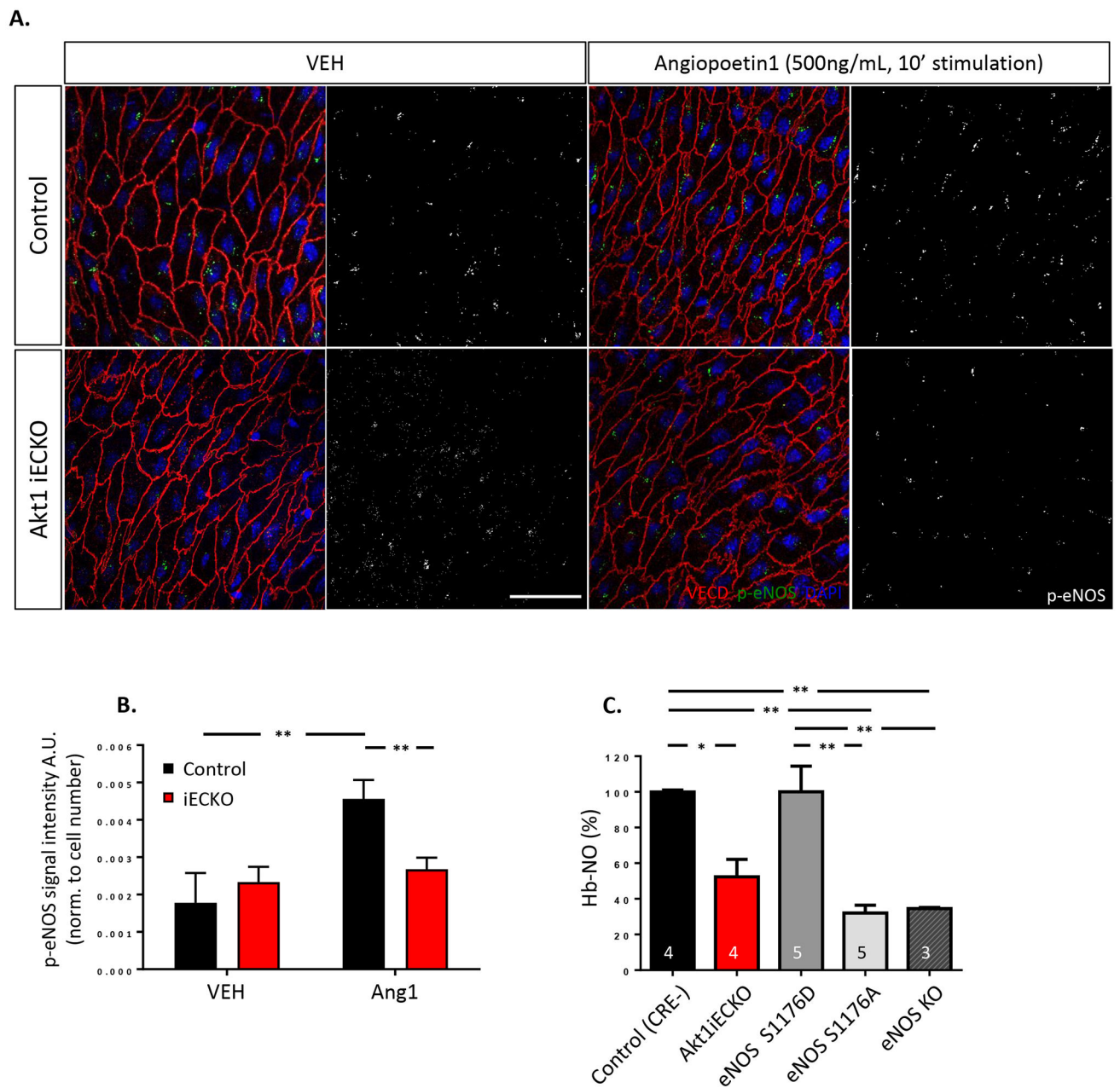
**A.**



**Figure 1. Endothelial loss of Akt1 impairs stimuli-induced eNOS phosphorylation and decreases NO bioavailability.**

(A) Isolated thoracic aorta segments from Akt1iECKO mice display decreased phospho-eNOS S1176 when treated with angiopoetin-1 ex vivo, quantified in (B). (C) Less hemoglobin-bound NO (NO-Hb) levels were detected in peripheral blood of Akt1iECKO adult mice when assessed by EPR techniques. eNOS mutant (S1176D and S1176A) and global eNOS KO mice were used as gating controls for NO-Hb levels. The number of mice for each genotype shown as indicated. All data are mean+SEM, n=3-6 mice per group, 2-6 images per mouse. * $p < 0.05$, ** $p < 0.01$.
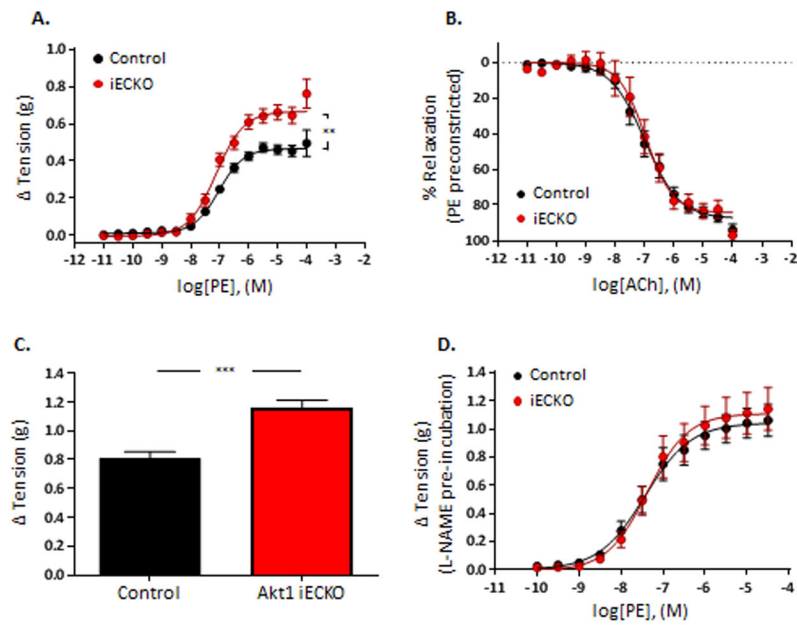
**Figure 2. Basal NO production in vessels requires endothelial Akt1.**
**(A)** Aortic rings from Akt1iECKO display increased sensitivity to phenylephrine (PE)-induced constriction, whereas **(B)** Ach-induced vascular relaxation is maintained, suggesting basal impaired NO production in Akt1iECKO mice. Aortic rings from Akt1iECKO mice display enhanced **(C)** KCl-induced (60mM) constriction, where **(D)** pre-incubation of vessels with L-NAME normalizes the differences in PE sensitivity. Data are mean± SEM, n=4-9 mice per group, 3-4 aortic rings/mouse. **p<0.01 ***p<0.001.
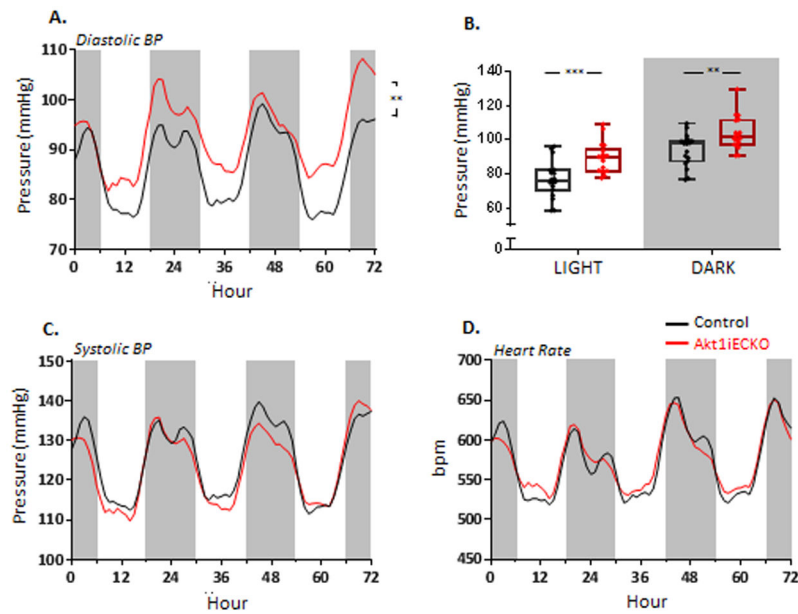
**Figure 3. Adult, endothelial-specific loss of Akt1 significantly increases diastolic BP.**
**(A)** Telemetry readings indicate an elevated diastolic pressure in free roaming, conscious Akt1iECKO mice. The increase occurred during the diurnal cycle **(B)**. The loss of Akt had no effect on systolic pressure, **(C)** or heart rate **(D)**, Data are mean ± SEM with n=6 mice per group. **p<0.01, ***p<0.001. Graphs illustrate a 3-day reading period where white and gray regions indicate light and dark cycles, respectively.
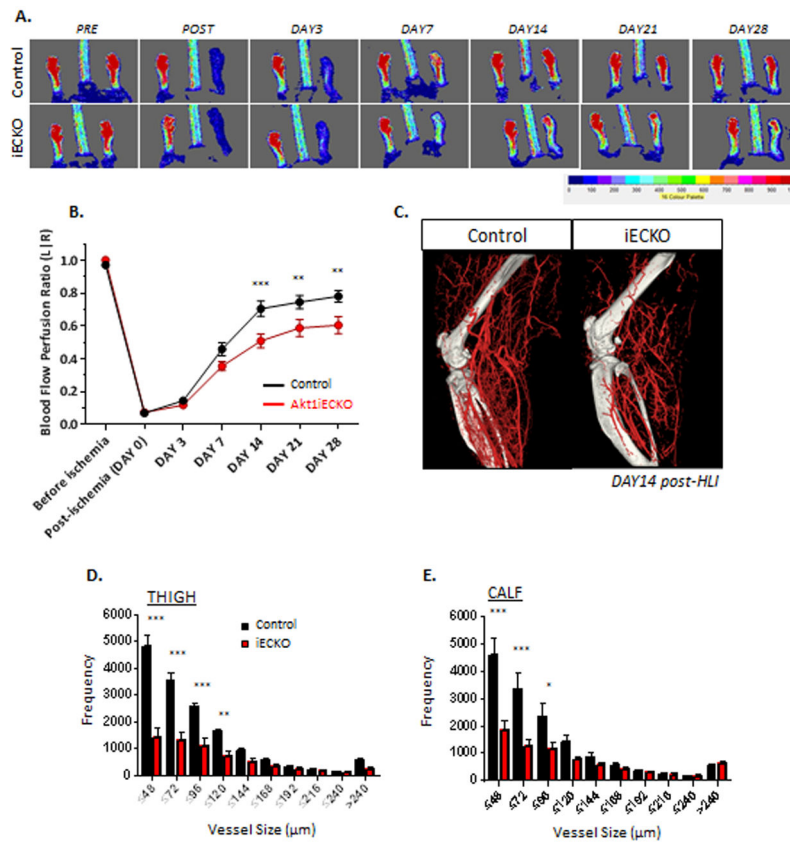
**Figure 4. Endothelial loss of Akt1 results in impaired blood flow recovery upon femoral artery ligation and reduced arteriogenesis.**

(**A**) Endothelial-specific loss of Akt1 results in impaired blood flow recovery in response to HLI 14 days post-ischemia. Relative blood flow perfusion ratio shown in (**B**). (**C**) Arterial phase microCT analyses at 14 days post-injury indicates significant reduction in small arteries/arterioles in thigh (**D**) and calf (**E**) regions in Akt1iECKO mice. Data are mean ± SEM, with n=5-6 mice/group. *p<0.05, **p<0.01, ***p<0.001.