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Androgen receptor promotes sex-independent angiogenesis in response to ischemia and is required for activation of vascular endothelial cell growth factor receptor signaling

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Abstract

Background—Hypoandrogenemia is associated with an increased risk of ischemic diseases. Since actions of androgens are exerted through androgen receptor (AR) activation, we studied hind limb ischemia in *AR* knockout (KO) mice to elucidate the role of AR in response to ischemia.

Methods and Results—Both male and female *ARKO* mice exhibited impaired blood flow recovery, more cellular apoptosis and a higher incidence of autoamputation after ischemia. In *ex vivo* and *in vivo* angiogenesis studies, *AR*-deficient vascular endothelial cells showed reduced angiogenic capability. In ischemic limbs of *ARKO* mice, reductions in the phosphorylation of the Akt protein kinase and endothelial nitric oxide synthase (eNOS) were observed despite a robust

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increase in hypoxia-inducible factor 1 α and vascular endothelial cell growth factor (*VEGF*) gene expression. In *in vitro* studies, siRNA-mediated ablation of AR in vascular endothelial cells blunted VEGF-stimulated phosphorylation of Akt and eNOS. Immunoprecipitation experiments documented an association between AR and kinase insert domain protein receptor (KDR) that promoted the recruitment of downstream signaling components.

Conclusion—These results document a physiological role of AR in gender-independent angiogenic potency and provide evidence for a novel cross-talk between androgen/AR signaling and VEGF/KDR signaling pathways.

Keywords

androgen receptor; angiogenesis; KDR; Akt; eNOS

Introduction

Cardiovascular disease (CVD) remains a major cause of human deaths, especially in males and gender differences in its onset and severity make it a key contributor to the life span gap between genders¹. Although the gender disparity in cardiovascular disease has been considered primarily as a reflection of estrogen-mediated protection against atherogenesis, conclusive clinical evidence for support of this hypothesis is lacking. In fact, two large-scale prospective randomized clinical trials showed no cardiovascular benefits of combined estrogen plus progestin therapy in postmenopausal women^{2,3}. On the other hand, increased understanding of the biological effects of androgens in the cardiovascular system has revealed a close relationship between a fall in male testosterone levels, called “andropause”, and an increase in cardiovascular events with aging. Many clinical studies have shown that hypoandrogenemia is associated with CVD⁴, cardiovascular death⁵, atherosclerosis⁶, metabolic syndrome, visceral fat obesity, type 2 diabetes^{7,8}. Testosterone replacement therapy has been shown to have beneficial effects on CVD risk factors, including visceral abdominal fat mass, insulin sensitivity, glycemic control, and hyperlipidemia⁹. We have also reported that serum levels of dehydroepiandrosterone sulfate, a major active androgen precursor, are inversely associated with carotid atherosclerosis¹⁰. However, the role of androgens in cardiovascular physiology and pathophysiology remains controversial, and some epidemiological studies have shown no obvious association of hypoandrogenemia with CVD^{11,12}. In addition, one study showed that testosterone administration to older men with a prevalence of chronic disease led to an increased risk of adverse cardiovascular events¹³. Thus, a better understanding of the molecular links between androgens and cardiovascular disease is warranted.

Androgen exerts various actions in its target organs, including the male genitalia, brain, bone, skeletal muscles, and endothelial cells, smooth muscle cells and fibroblasts in the vascular system¹⁴. Biological activities of androgen are predominantly mediated through the transcriptional control of target genes by genomic actions of the nuclear androgen receptor (AR)¹⁵. Androgen also exerts nongenomic actions by interacting with multiple signaling pathways, independent of transcriptional control^{16,17}. Such nongenomic effects of androgen may occur through AR in the cytoplasm to induce the rapid activation of kinase-signaling cascades¹⁷.

In order to clarify the pathophysiological role of AR in various target organs *in vivo*, we examined AR knockout (KO) mice generated by a Cre-loxP system. Male ARKO mice manifest late-onset obesity¹⁸, high-turnover osteopenia¹⁸, impaired brain masculinization¹⁹, and aberrant adiponectin expression²⁰. In addition, we have demonstrated that AR activation plays a pivotal role in not physiological cardiac growth, as

well as protection from pathological cardiovascular remodeling induced by angiotensin II^{21, 22} and cardiotoxicity induced by the anticancer agent doxorubicin²³.

Although skeletal muscle ischemia caused by peripheral arterial disease (PAD) and/or thrombotic disorders is also a critical problem in elderly subjects with andropause²⁴, it has been unclear whether AR plays a distinct pathophysiologic role in response to skeletal muscle ischemia. In order to clarify this issue, we studied the role of AR in response to ischemic injury using a hind limb ischemia model in *ARKO* mice. The results indicated gender-independent novel protective mechanisms of AR actions against ischemic injury.

Methods

Animals and Experimental Protocol

Male and female *ARKO* mice were generated by targeted disruption of the *AR* gene using the Cre-loxP system as previously described^{18, 19, 25, 26}. Male *ARKO* mice exhibited testicular feminization, and reduction in levels of serum gonadal androgens (testosterone and dihydrotestosterone), whereas serum adrenal androgen and estrogen levels remained normal¹⁸. Female *ARKO* mice have normal serum hormone levels, including 17 β -estradiol, progesterone, testosterone, luteinizing hormone and follicle-stimulating hormone²⁶. In the present study, we used 25-week-old male and female *ARKO* mice and age-matched littermate male and female wild-type (WT) mice. Mice were backcrossed for 10 generations with the C57BL/6J strain. To create hind limb ischemia in these mice, the proximal portion of the femoral artery and the distal portion of the saphenous artery were ligated and then excised^{27, 28}. We performed the following experimental procedures: macroscopic evaluation of ischemic severity, laser speckle blood flow analysis, immunohistochemistry, real-time PCR analysis, Western blot analysis, aortic ring assay, *in vivo* angiogenesis assay and bone marrow transplantation, siRNA experiments, immunoprecipitation, *in situ* proximity ligation assay. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, The University of Tokushima Graduate School of Health Biosciences. Details of the experimental procedures can be found in the online supplement.

Statistical analysis

Values for each parameter within a group are expressed as dot plots with mean bars. For comparisons of quantitative data among groups, statistical significance was assessed by the Kruskal-Wallis test. The Bonferroni-corrected Mann-Whitney U test or Dunn's test was used for multiple comparisons. For comparison of time-dependent changes among groups, statistical significance was assessed by linear mixed effects regression analysis. Limb survival rate was assessed by the log-rank test. These analyses were performed by using Excel (Microsoft Office Excel 2007; Microsoft, Richmond, CA), PASW Statistics 18.0 (IBM SPSS Japan Inc., Tokyo, Japan), GraphPad Prism6 (GraphPad Software, San Diego, CA) and JMP (SAS Institute Japan Ltd., Tokyo, Japan). Statistical significance was set at $P < 0.05$.

Results

Increased incidence of autoamputation in *ARKO* mice after hind limb ischemia

In order to elucidate the role of AR in ischemic response, we created a model of hind limb ischemia induced by surgical arteriectomy of the right femoral artery in *ARKO* and control mice. Three weeks after surgery, necrotic changes were macroscopically evaluated. Both male and female WT mice showed limited necrosis of toes. In contrast, hind limb autoamputations occurred in more than half (53%) of male *ARKO* mice at day 21 after

ischemic surgery (Figure 1A and B). Interestingly, female *ARKO* mice also demonstrated extensive necrosis, leading to autoamputation at nearly the same frequency as male *ARKO* mice (42%) (Figure 1C).

Accelerated cellular apoptosis of ischemic skeletal muscle in *ARKO* mice

Ischemia-induced cellular apoptosis of skeletal muscle was examined in both genders of WT and *ARKO* mice by TUNEL staining. The number of TUNEL-positive cells in ischemic skeletal muscle was significantly increased in both male and female *ARKO* mice compared to those in respective male and female WT mice (Figure 2A-D). Immunohistochemical examination confirmed that the majority of TUNEL-positive cells were skeletal myocytes (black arrows), but apoptotic endothelial cells were also observed (blue arrows) (Figure 2E). Next, the expression ratio of *Bcl-2*-to-*Bax*, key factors of the apoptotic signal pathway was analyzed in ischemic muscle. Real-time PCR analysis and Western blot analysis using tissue homogenates of ischemic adductor muscles obtained from male and female *ARKO* mice at 1 day after ischemia showed a lower *Bcl-2* (*Bcl-2*)-to-*Bax* (*BAX*) expression ratio than those in respective male and female WT mice (Figure 2F-K). In male mice, the *Bcl-2*-to-*Bax* ratio at day 1 decreased on mRNA level, though the protein ratio of those proteins was different result. These results indicate the possibility that there is a gender difference in the stability of *Bax* and *Bcl2* mRNAs. Taken together, these findings indicate that the severity of ischemia-induced cellular apoptosis leading to autoamputation of the hind limb is more accelerated in *ARKO* mice than those in WT mice regardless of gender.

Impaired blood flow recovery with reduced capillary density in surviving skeletal muscle of *ARKO* mice after ischemia

Blood flow recovery of ischemic skeletal muscle in both male and female WT mice and that of surviving ischemic skeletal muscle in both male and female *ARKO* mice were evaluated for 3 weeks by laser speckle blood flow (LSBF) imager. In male and female WT mice, blood flow fell precipitously until one day after ischemic surgery; however, blood flow recovered promptly and had returned to almost non-ischemic hind limb levels at day 21. In contrast, the recovery of blood perfusion in surviving ischemic hind limbs of male and female *ARKO* mice was poor throughout the follow-up period, and the ratio of ischemic to non-ischemic laser speckle blood flow was persistently lower in *ARKO* mice than in WT mice regardless of sex (Figure 3A, B, E and F). At 3 weeks after surgery, capillary vessels identified by CD31 staining were markedly increased in ischemic muscle of male and female WT mice, whereas this compensatory response against ischemia was almost completely abrogated in male and female *ARKO* mice (Figure 3C, D, G and H). In addition, smaller numbers of α smooth muscle actin (α SMA)-stained cells indicating vascular pericytes, were observed in male and female *ARKO* mice after ischemia (supplemental figure 1).

Attenuated angiogenic potency in AR-deficient vascular endothelial cells

In order to clarify the influence of AR deficiency on angiogenic potency of vascular endothelial cells, aortic ring assay and *in vivo* angiogenesis assay were performed (Figure 4A and B). Figure 4A shows representative photos and quantitative results of microvascular sprouting at day 7 after aortic ring implantation. We found that the number of sprouting microvessels and length of microvessels were significantly reduced in aortas from male *ARKO* mice compared to those in aortas from male WT mice. Finally, the angiogenesis assay involving the implantation of tubes containing basement membrane extract with the angiogenic factors also showed reduced vessel invasion when the tubes were implanted in male *ARKO* mice compared to that in tubes implanted in male WT mice (Figure 4B). These

results are consistent with the notion that AR-deficient vascular endothelial cells have reduced angiogenic potency.

Bone marrow cells are not involved in impaired angiogenesis of male ARKO mice after ischemia

Bone marrow (BM)-derived progenitor cells have been shown to participate in revascularization in ischemic tissues²⁹. In this regard, it has been proposed that the mobilization of angiogenic progenitors contributes to the effects of androgens on the revascularization process³⁰. To determine whether BM-derived cells are involved in impaired angiogenesis of ARKO mice, we performed BM transplantation between male WT and male ARKO mice. As shown in Figure 4C and D, limb survival rate and blood flow recovery from ischemia were not decreased in male WT mice receiving male ARKO BM cells. In addition, male WT BM cells transplanted into male ARKO mice did not prevent autoamputation in these mice after hind limb ischemia (Figure 4C). Laser speckle imaging also demonstrated that male WT BM cell transplantation failed to restore the impaired blood flow recovery in surviving skeletal muscle of male ARKO mice after ischemia (Figure 4D). These results indicate that the impaired blood flow recovery from ischemic stress in male ARKO mice is not due to an impaired angiogenic potency of BM-derived progenitor cells of these mice.

Aberrant gene expression levels of angiogenesis-related factors in ARKO mice after hind limb ischemia

Gene expression levels of angiogenesis-related factors were analyzed in the adductor muscle by real-time PCR analysis. Under non-ischemic conditions, there were no significant differences in gene expression levels of proangiogenic factors, including hypoxia inducible factor 1 α (*Hif1a*), vascular endothelial growth factor A (*Vegfa*), fibroblast growth factor 2 (*Fgf2*) and the VEGF receptor, *Kdr* between both sexes of WT and ARKO mice (Figure 5A and B). However, following surgery-induced ischemia, expression levels of *Hif1a*, *Vegfa* and *Fgf2* were prominently augmented in male and female ARKO mice compared to those in respective male and female WT mice. The ischemia-reduced elevation of *Kdr* mRNA levels was attenuated in male ARKO mice but not female ARKO mice (Figure 5A and 5B).

Reduced regulation of cell survival and proangiogenic factors in ARKO mice

Our previous studies demonstrated that extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt-eNOS signaling pathways are activated by the non-genomic actions of AR stimulation²¹⁻²³. Therefore, we investigated the role of the androgen/AR system in the regulation of these pathways in ischemic limbs because they are involved in cell survival and angiogenesis. Ischemic muscles of male and female ARKO mice showed significant attenuation of ERK1/2, Akt and eNOS phosphorylation compared with those in respective male and female WT mice (Figure 6A and 6B). Since both Akt and ERK1/2 activation promote cell survival and since activation of Akt and eNOS enhances angiogenesis^{31, 32}, the acceleration of ischemia-induced cellular apoptosis and impaired angiogenesis in both male and female ARKO mice may be explained, at least in part, by the reduced activation of these factors.

AR knockdown in HUVECs blunts activation of the VEGF receptor signaling pathway

In order to determine whether reduced activation of the Akt-eNOS pathway in ischemic muscles of ARKO mice was associated with a blunted responsiveness of endothelial cells to VEGF stimulation, we next examined VEGF-induced activation of the Akt-eNOS signaling pathway in vascular endothelial cells with or without siRNA-mediated AR knockdown. (In this study, the siRNA reduced AR mRNA levels to $17.0 \pm 1.8\%$ of the control and reduced

AR protein levels to $20.8 \pm 2.0\%$ of the control.) VEGF stimulation in the presence of 5 α -dihydrotestosterone (DHT) enhanced Akt and eNOS phosphorylation in control HUVEC cultures. In contrast, VEGF-stimulated Akt and eNOS phosphorylation was blunted in HUVECs with AR knockdown (Figure 7A). These results indicate that AR-mediated signaling potentiates VEGF-mediated activation of the Akt-eNOS pathway in vascular endothelial cells.

Ligand-bound AR promotes complex formation with KDR, Src and PI3K

Stimulation by VEGFs rapidly induces KDR dimerization and autophosphorylation, followed by recruitment and activation of Src and phosphoinositide-3-kinase (PI3K)³³. AR is also shown to recruit Src and activate the mitogen-activated protein kinase (MAPK) pathway³⁴, and activate the PI3K-Akt cascade³⁵, leading to cell survival and proliferation. Since we found that VEGF-stimulated Akt and eNOS phosphorylation was blunted by AR deficiency, we examined whether AR associates with VEGF receptor and affects its downstream signaling pathway in endothelial cells. Immunoprecipitation of HUVEC lysates using an anti-AR antibody showed an association of AR with KDR, PI3Kp85, and Src that was augmented by DHT supplementation (Figure 7B). Moreover, immunoprecipitation experiments using an anti-KDR antibody in the presence of DHT and VEGF revealed that AR, PI3Kp85 and Src were associated with KDR, whereas AR knockdown attenuated the associations between PI3Kp85, Src and KDR (Figure 7C). In order to clarify the association between KDR and AR more clearly, we employed the HaloTag technology³⁶. Exogenous AR-HaloTag fusion proteins and HaloTag control proteins were expressed in endothelial cells, and then a HaloTag pull-down assay was performed on cell lysates to identify candidate AR-bound proteins by Western blot analysis. These results showed that DHT supplementation promotes the associations between AR-HaloTag fusion protein and KDR, PI3Kp85 and Src, while those protein associations are diminished in the absence of DHT (Figure 7D). Taken together, these results are consistent with the notion that AR associates with KDR and promotes associations between KDR, Src and PI3K.

Ligand-bound AR associates with KDR at the plasma membrane and cytoplasm of vascular endothelial cells in situ

In order to visualize the association between AR and KDR in vascular endothelial cells, we used an *in situ* proximity ligation assay (PLA), a method that is used for detecting and imaging protein associations in both tissue sections and cultured cells *in vitro*³⁷. The *in situ* PLA revealed modest AR/KDR proximity signal in HUVECs under baseline conditions (Figure 7E, left panel). VEGF stimulation alone led to a slightly increased the AR/KDR proximity signal (Figure 7E, middle panel). However, the combination of VEGF and DHT led to markedly greater AR/KDR complex formation (Figure 7E, right panel and 7F). These results indicate that DHT promotes the association between AR and KDR outside the nucleus in vascular endothelial cells, consistent with the findings of enhanced VEGF actions under these conditions.

Discussion

In the present study, we showed that AR is essential for robust revascularization in response to ischemia in both male and female mice. Male and female ARKO mice manifested hindlimb autoamputation after ischemic surgery, and AR-deficiency was associated with greater of ischemia-induced cellular apoptosis of myofibers and endothelial cells. Our study also documents a hitherto unrecognized cross-talk mechanism between androgen/AR and VEGF/KDR signaling pathways. Collectively, these data provide a molecular rationale for why “andropause” individuals are at greater risk for ischemic disease.

Pathophysiological roles of AR in females are largely unknown. Shiina et al. have demonstrated that eight-week-old female *ARKO* mice manifest reduced ovarian follicle numbers, impaired mammary development, and reduced pups numbers²⁶. In addition, they have shown that forty-week-old *ARKO* mice have no ovarian follicles leading to infertility with aberrant gene expression levels of folliculogenesis related factors²⁶. These results and our present study suggest that liganded and/or unliganded AR activation might partially participate in biological homeostasis of multiple organs dependent and/or independent of sex and/or estrogen.

Aspects of our study are consistent with a prior report by Sieveking et al. showing that androgens have a positive effect on angiogenesis³⁰. For example, they reported that castration led to reduction in ischemia-induced angiogenesis. However, utilization of the *ARKO* mouse in the current study has enabled us to reach additional conclusions. For example, Sieveking et al. concluded that androgens promote angiogenesis in male but not female mice after ischemia³⁰. In contrast, we show that AR-deficiency impairs ischemia-induced angiogenesis in both male and female mice. There are a number of possible reasons for this apparent discrepancy. One possibility is the presence (*ARKO* mice) or absence (ovariectomised WT mice) of estrogen in female mice, which can influence a revascularization response^{38,39}. Alternatively, the presence (ovariectomised WT mice) or absence (*ARKO* mice) of AR in female mice could account for the difference in these findings. It has been reported that unliganded nuclear receptors, including thyroid hormone receptor, estrogen receptor, progesterone receptor and vitamin D receptor have considerable biological actions⁴⁰⁻⁴³, we speculate that unliganded AR also plays a role in angiogenesis after ischemia regardless of gender. Further experiments are required to clarify these issues.

Sieveking et al.³⁰ also reported that treatment with androgen promoted the mobilization of bone marrow progenitor cells that could theoretically promote revascularization via paracrine secretion of angiogenic factors. Similarly, it has been reported that estrogen can mobilize bone marrow-derived angiogenic cells that can contribute to the revascularization process⁴⁴. However, as reported here, limb reperfusion and protection from necrosis were not impaired in male WT mice receiving male *ARKO* bone marrow cells, nor were these parameters improved when male *ARKO* mice were transplanted with male WT bone marrow. Thus, while sex hormones can alter the mobilization of cell populations from bone marrow, the transplantation studies conducted in the current study show that AR-deficient bone marrow progenitor cells have little or no impact on the limbs subjected to ischemia.

AR-deficiency led to molecular changes associated with diminished cell viability in the ischemic limb, including a reduction in the Bcl-2-to-Bax ratio and inactivation of ERK1/2 and Akt signaling pathways. The proapoptotic protein BAX plays a critical role in the intrinsic apoptotic pathway, and Bcl-2 forms heterodimers with BAX and prevents its insertion into the mitochondrial membrane. Therefore, the Bcl-2-to-BAX ratio is critical for determination of the apoptotic threshold⁴⁵. Lin et al. reported that AR is able to counteract BAX-mediated apoptosis in the prostate gland in an androgen-dependent and independent manner⁴⁶. In addition, our previous study showed that the Bcl-2-to-BAX ratio after treatment with doxorubicin, a cytotoxic agent, was lower in male *ARKO* mice than in male WT mice²³. These results are consistent with the assumption that AR-mediated signaling protects against cellular apoptosis.

The ERK1/2 signaling pathway promotes cell survival by a dual mechanism: inactivation of components of the cell death machinery and enhancement of transcription of pro-survival genes⁴⁷. Additionally, Akt has been shown to be a cytoplasmic serine/threonine kinase that contributes to the control of apoptosis machinery and cellular metabolism⁴⁸. We previously reported that angiotensin II-induced activation of ERK1/2 is abrogated in aortic tissues and

cardiac myocytes of male *ARKO* mice, leading to aberrant vascular remodeling and left ventricular systolic dysfunction with extended cardiac fibrosis^{21, 22}. Moreover, we have reported that oxidative stress and apoptosis of cardiomyocytes were enhanced in male *ARKO* mice by doxorubicin treatment with reduced cardiac mitochondrial transcription factor A expression and Akt phosphorylation²³. Consistent with these observations, the present study demonstrated that AR deficiency accelerates cellular apoptosis through reduced Bcl-2-to-BAX ratio and impaired phosphorylation of ERK1/2 and Akt under the ischemic stress regardless of sex.

The PI3K/Akt pathway is activated by VEGF and is capable of modulating cell survival, migration, tube formation, and nitric oxide (NO) release⁴⁹. In fact, studies using mice with Akt 1 gene deletion demonstrated that Akt 1 is essential for promoting ischemic and VEGF-initiated postnatal angiogenesis and vascular maturation^{50, 51}. It is well known that Akt activates eNOS and enhances NO production³² and that release of NO from vascular endothelial cells is essential for promoting angiogenesis and collateral vessel remodeling in response to ischemia²⁸. Therefore, it is reasonable to assume that the reduced Akt-eNOS phosphorylation in ischemic skeletal muscle of *ARKO* mice is closely associated with not only cellular apoptosis but also suppressed angiogenesis, with disturbed vascular maturation and collateral vessel remodeling. With regard to the relationship between Akt-eNOS signaling and AR signaling pathways, we have previously demonstrated that AR deficiency causes a reduction in eNOS expression and phosphorylation, leading to attenuation of NO bioavailability and acceleration of vascular remodeling under the condition of angiotensin II excess²². These observations led us to conclude that AR is required for promoting angiogenesis partly through activation of the PI3K/Akt-eNOS signaling pathway in response to ischemic stress.

In the present study, the revascularization impairment in both male and female *ARKO* mice was associated with a rapid elevation in the levels of angiogenic growth factors (*Vegfa* and *Fgf2*), as well as an increase in *Hif1a* expression. The mechanism whereby the expression of *Vegfa* and *Hif1a* genes was rapidly enhanced after ischemia is not clear. However, the observation that ischemic limbs from *ARKO* mice exhibited marked edema and a dusky red-colored skin appearance on day 1 after surgery are consistent with exaggerated ischemia in the operated limbs of *ARKO* mice. Since previous studies showed that castration rapidly reduces blood flow to the rat ventral prostate gland with acceleration of epithelial cellular apoptosis and tissue hypoxia^{53, 54} and since we have reported that AR deficiency reduces nitric oxide bioavailability²², lack of androgen action may lead to prominent tissue hypoxia due to circulatory insufficiency. Although further investigations are needed to clarify this issue, we speculate that the enhanced *Hif1a* and *Vegfa* expression levels reflect a more severe acute ischemic condition in *ARKO* mice than in WT mice. Interestingly, the enhanced gene expression levels of *Kdr* as compensatory response against ischemic stress was attenuated in male *ARKO* mice but not in female *ARKO* mice. Activation of the VEGF-VEGF receptor axis is a critical step for the initiation and development of angiogenesis. VEGF primarily utilizes its receptor KDR, also known as VEGFR-2 or FLK-1, to induce angiogenic responses mediated by subsequent activation of signaling cascades such as PIK3-Akt and MAPK⁵². Given the critical role of KDR signaling in angiogenesis, regulation of KDR is pivotal for the regulation of angiogenesis. Sieveking et al. reported that DHT induced dose-dependent increases in the mRNA expression of *KDR* in male human vascular endothelial cells³⁰. Their results as well as our present observations support the notion that AR activation is required for compensatory up-regulation of *Kdr* gene expression in response to ischemia at least in males. Although the relationship between sex hormones, AR and *Kdr* gene expression in female mice have remained unclear, the role of AR in female ischemic muscle may be different from that in male ischemic muscle in terms of *Kdr* expression. Sieveking et al. reported that androgens up-regulate expression levels of *Hif1a*

and VEGF; however, we found that *in vivo* *Hif1a* and *Vegfa* expression levels are augmented more in ARKO mice than in WT mice after ischemia in both males and females. While their experiments were performed using cultured endothelial cells *in vitro*, we determined gene expression levels of *Hif1a* and *Vegfa* *in vivo* in ischemic tissues including skeletal muscle, vasculature, bone, lymphatic tissue and nerves. Because skeletal muscle is an abundant source of VEGF, the discrepancy between their results and ours may be due to differences in experimental conditions in the two studies.

In the present study, we also found that VEGF-stimulated Akt and eNOS phosphorylation was blunted by AR deficiency and that AR associated with KDR through recruitment of signaling molecules including PI3Kp85 and Src. These results suggest a close interplay between AR and KDR. Further studies will be required to document whether the interaction between AR and KDR is direct, or whether other proteins are required for the formation of this complex. In this regard, Sun et al. showed that a ternary complex forms among AR, PI3Kp85 and Src that is essential for androgen-stimulated activation of PI3K/Akt and MAPK³⁵. Therefore, there is a possibility that AR and KDR share these same signaling molecules and that AR activation and association with KDR play an important role in full activation of the KDR signaling pathway in response to ischemia. The present observations provide evidence for a novel cross-talk mechanism between androgen/AR signaling and VEGF/KDR signaling pathways as illustrated in Figure 8 and suggest a therapeutic potential of androgen for ischemic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Akt	Serine/threonine protein kinase
AR	androgen receptor
ARKO	AR knockout
BM	bone marrow
CVD	cardiovascular disease
DHT	dihydrotestosterone

eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinase1/2
FGF2	fibroblast growth factor2
HIF1α	hypoxia-inducible factor 1 α
KDR	kinase insert domain protein receptor
LSBF	laser speckle blood flow
MAPK	mitogen-activated protein kinase
PAD	peripheral arterial disease
PI3K	phosphoinositide-3-kinase
PLA	proximity ligation assay
VEGF	vascular endothelial cell growth factor

Clinical Perspectives

In addition to menopause, andropause has been well recognized as a risk factor for cardiovascular diseases (CVDs). Therefore, gender-specific clinical and experimental data are important in delineating gender-dependent differences in the incidence of and vulnerability to CVDs. Here, we show that androgen receptor (AR) activation plays an important role in angiogenesis after ischemia regardless of genders. This study identifies the function of the AR in the compensatory response to ischemia *in vivo*, and defines that its mechanism of action involves the association of AR with a VEGF receptor and activation of pro-angiogenic downstream pathways. Although beneficial anabolic effects of androgens on various tissues including bone, skeletal muscles, and central nerves system are well documented, clinical use of androgens has been limited because of their undesirable side effects. These side effects include the increased risk of prostate cancer in men, and hirsutism in women. Recently, selective androgen receptor modulators (SARMs) have been under clinical development, and trials are under way in patients with sarcopenia and cachexia, conditions that are closely associated with cardiovascular complications. Based upon the present study, we speculate that the appropriately targeted regulation of AR function in ischemic tissues may lead to novel therapeutic approaches for the treatment of CVDs in both sexes.

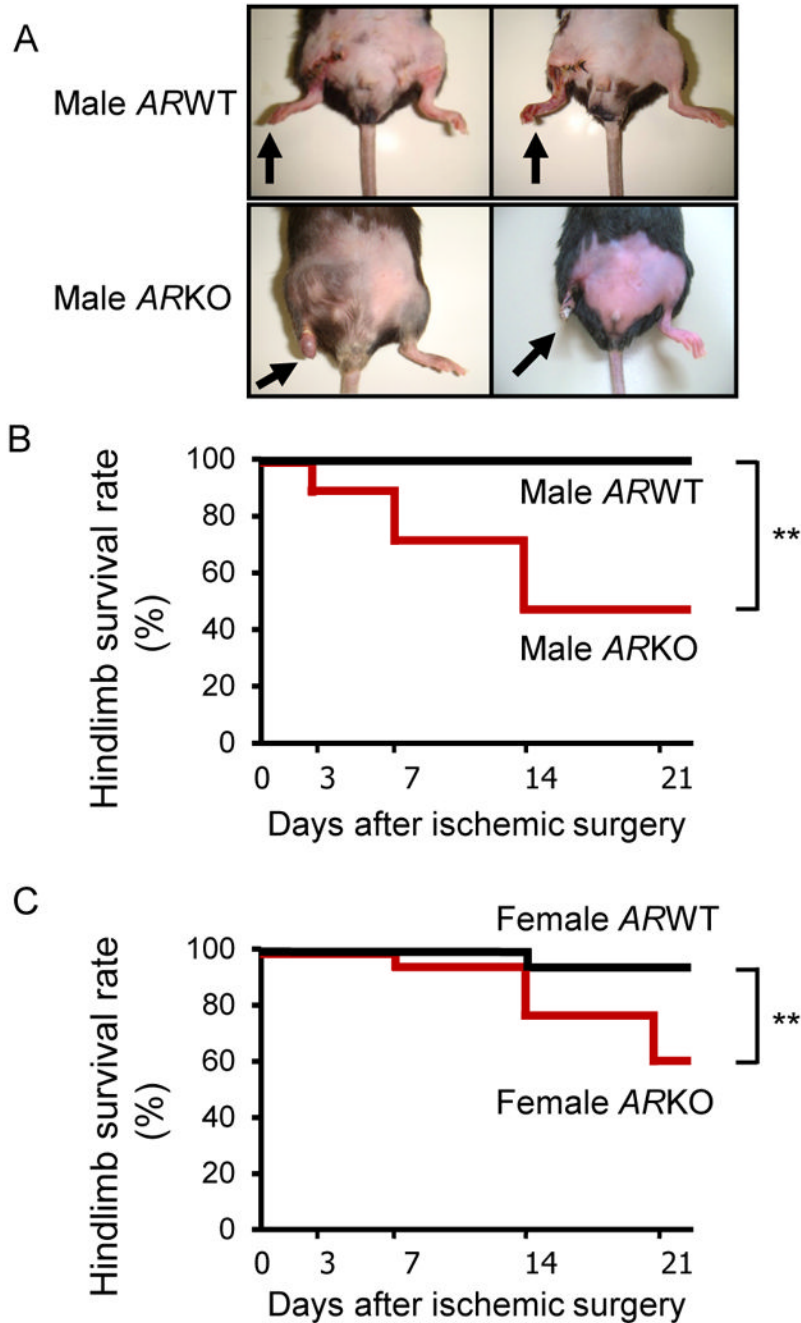


Figure 1. Increased hind limb autoamputation in both male and female ARKO mice after femoral arteriectomy

(A) Representative photographs of hind limbs of male WT and male ARKO mice at day 21 after ischemic surgery. (B) Limb survival rate after ischemic surgery in male WT and ARKO mice. Autoamputation of the hind limb was defined as extended necrosis above the knee. $n=17$ in each male mice group. $**P<0.01$, male ARWT vs. male ARKO using Log-rank test. (C) Limb survival rate after ischemic surgery in female WT and ARKO mice. $n=25$ in the female WT mouse group and $n=12$ in the female ARKO mouse group. $**P<0.01$, female ARWT vs. female ARKO using Log-rank test.

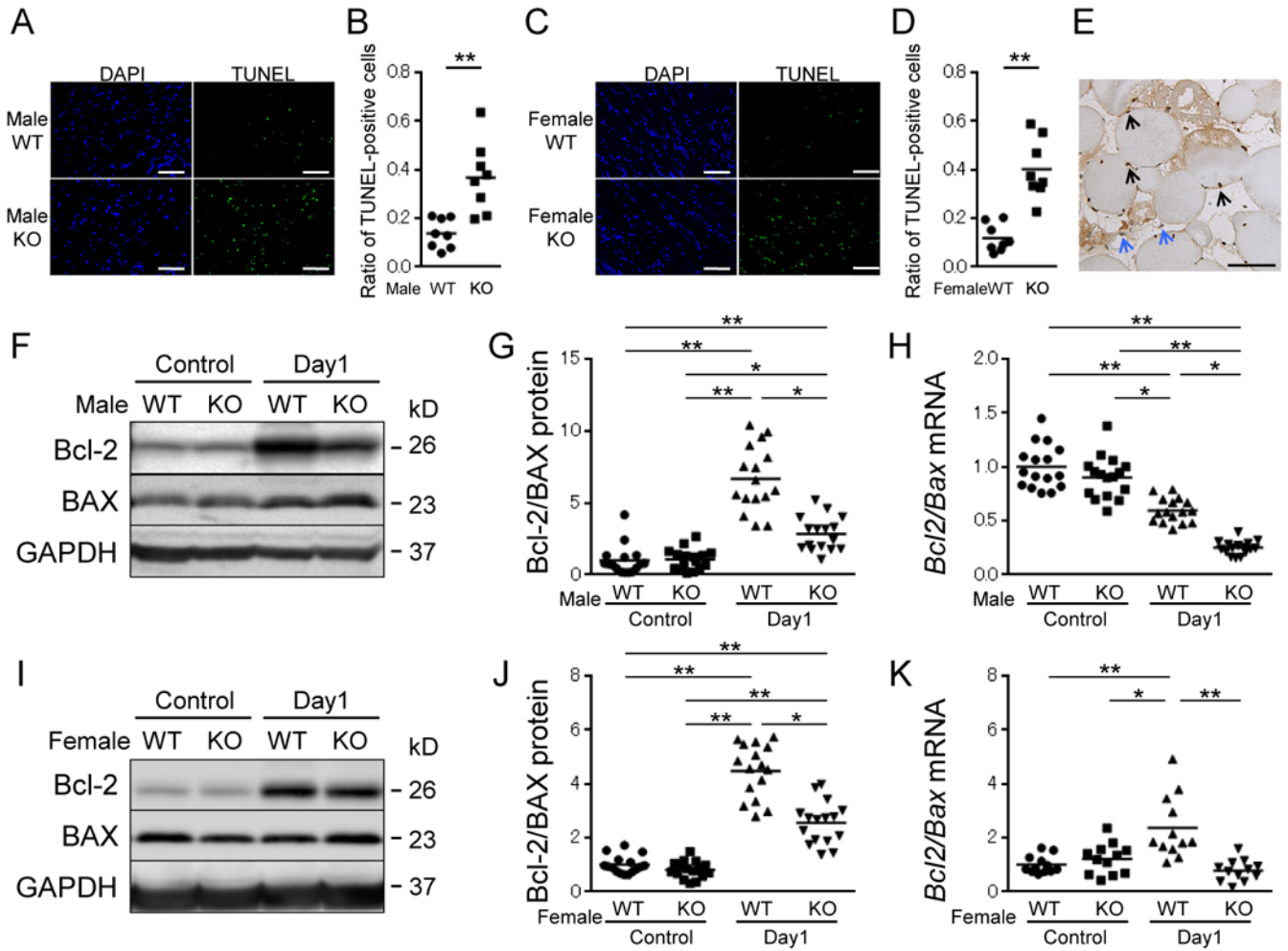


Figure 2. Accelerated cellular apoptosis in ischemic skeletal muscles of both male and female ARKO mice

(A and C) Immunofluorescence of TUNEL staining in ischemic adductor muscle of male WT and ARKO mice (A) or female WT and ARKO mice (C) at day 1 after ischemia. Scale bar indicates 200 μm. (B and D) Ratio of TUNEL-positive cells to total nuclei in ischemic muscle of male mice (B) and female mice (D). *n*=8 in each group. (E) TUNEL staining by an enzyme antibody technique in ischemic muscle of male ARKO mice at day 1. Black arrows denote nuclei of skeletal muscle cells. White arrows denote nuclei of endothelial cells. Scale bar indicates 50 μm. (F, G, I and J) Protein levels of Bcl-2, BAX and Bcl-2-to-BAX ratio by Western blot analysis in muscle of both sexes of WT and ARKO mice at before surgery (control) and day 1 after ischemia. The value of WT control was set as 1. (H and K) mRNA expression levels of Bcl-2-to-Bax ratio by quantitative real-time PCR in ischemic muscle of both sexes of WT and ARKO mice before surgery (control) and at day 1 after ischemic surgery. *n*=16 in each group in Figures G, H and J, *n*=12 in each group in Figure K. **P*<0.05, ***P*<0.01 using the Bonferroni-corrected Mann-Whitney U test following the Kruskal-Wallis test. Bars represent mean values in each group.

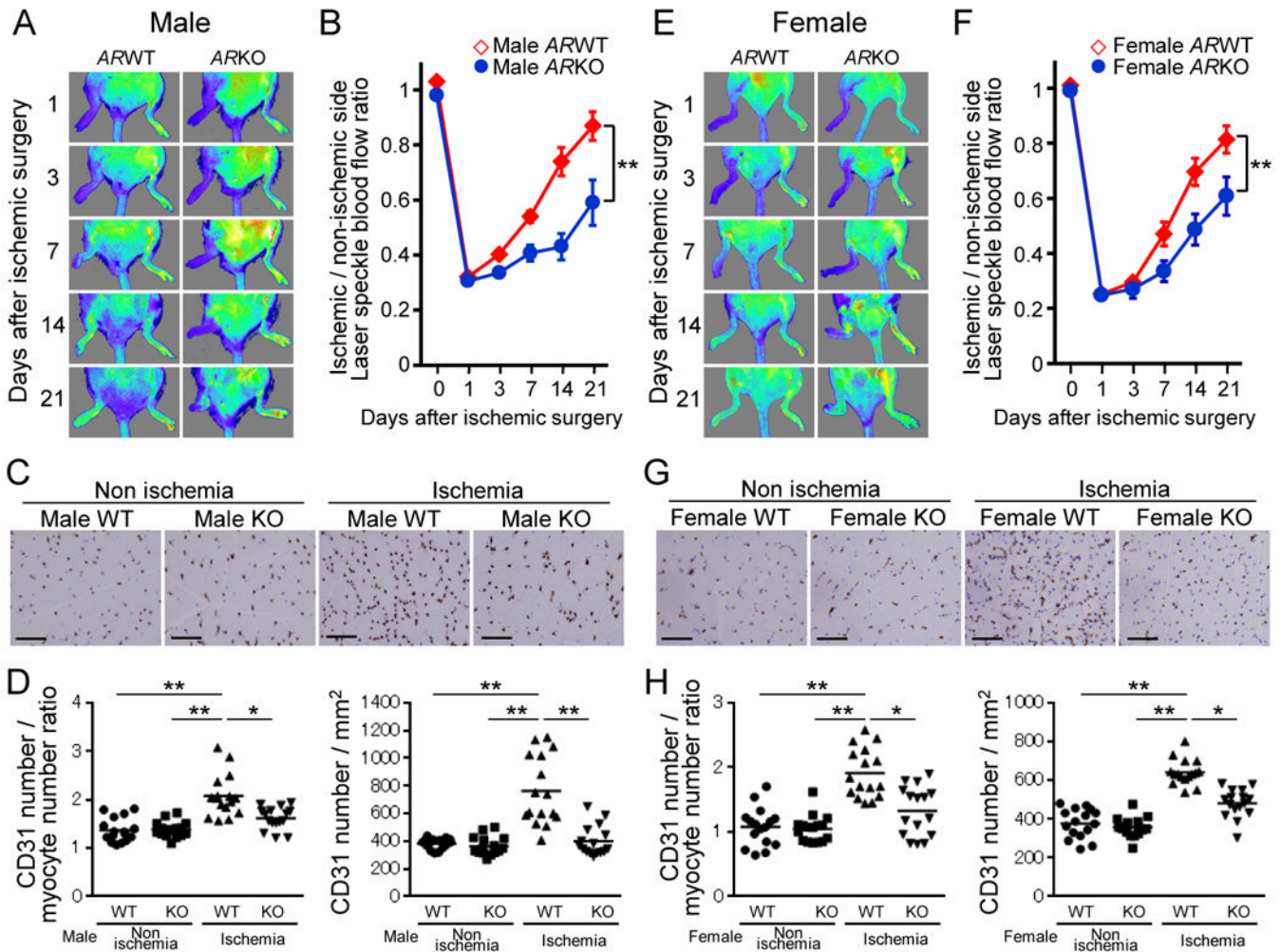


Figure 3. Impaired blood flow recovery and reduced capillary density in both male and female ARKO mice after ischemia

(A and E) Representative laser speckle blood flow (LSBF) images of the ischemic hind limb in WT and ARKO mice during the experimental period. A low perfusion signal is indicated as dark blue and according to increase in perfusion signals, the colors are indicated as green, yellow and red in series. (A) Male WT mice ($n=17$) and male ARKO mice (surviving hind limbs, $n=8$). (E) Female WT mice (surviving hind limbs, $n=16$) and female ARKO mice (surviving hind limbs, $n=7$). (B and F) Cumulative results are shown as ratio of blood flow in the ischemic limb to that in the nonischemic limb at each time point (B: male mice, F: female mice). $**P<0.01$ using a linear mixed effects regression analysis. Error bars represent \pm SEM. (C and G) CD31 immunohistochemical staining to determine capillaries in ischemic and nonischemic thigh adductor muscles in WT and ARKO mice at day 21 after surgery (C: male mice, G: female mice). Scale bar indicates 100 μm . (D and H) Quantification of capillary density is expressed as CD31-positive number per myocyte number or per square millimeter in WT and ARKO mice (D: male mice, H: female mice). $n=16$ in each group. $*P<0.05$, $**P<0.01$ using Dunn's test following the Kruskal-Wallis test. Bars represent mean values in each group.

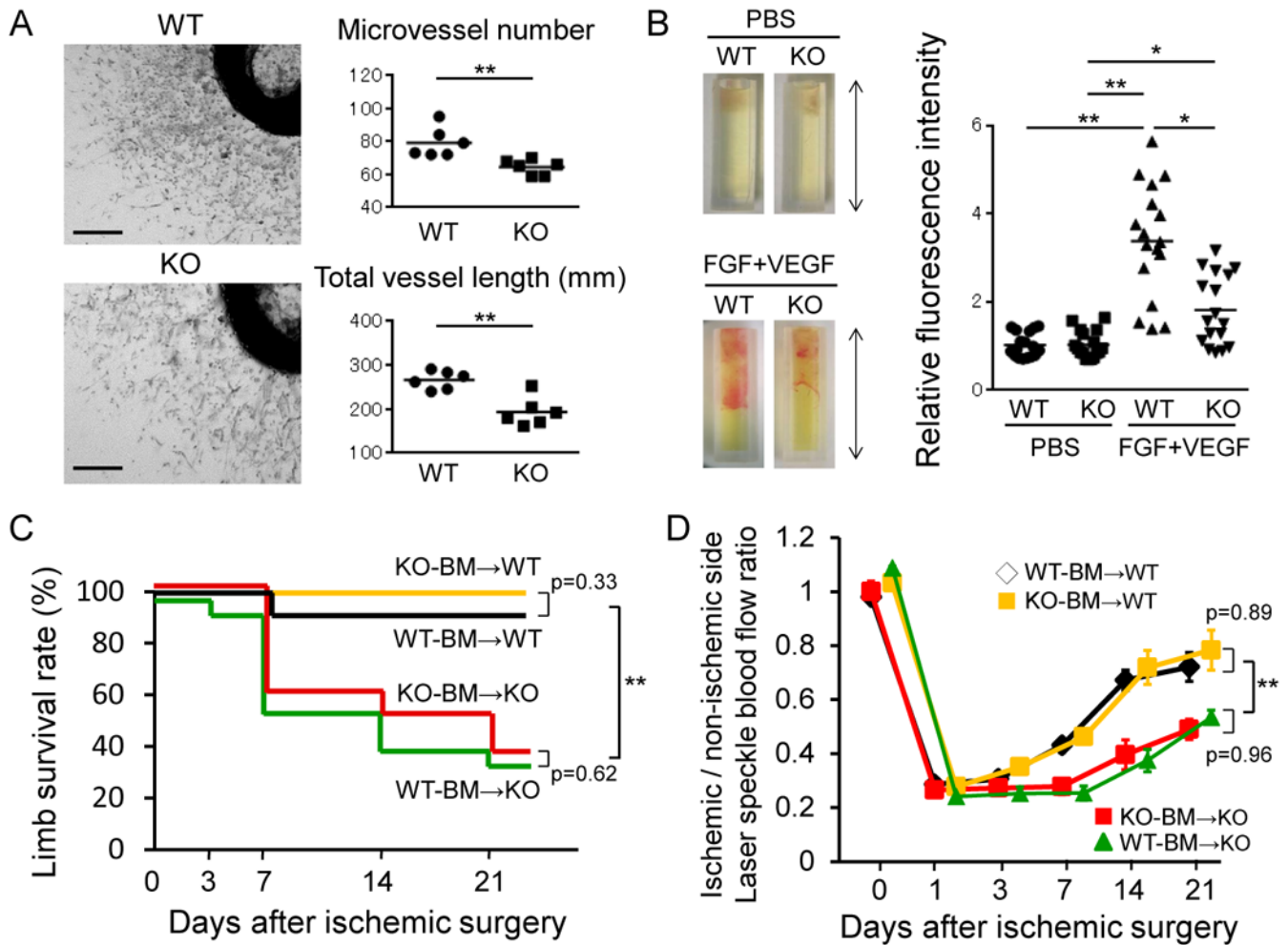


Figure 4. Attenuated angiogenic potency in endothelial cells but not in bone marrow progenitor cells of male ARKO mice

(A) Aortic ring assay. (A, left) Micrographs of representative aortic ring microvessels from male WT and male ARKO mice grown in the presence of growth factors at day 7. Scale bar indicates 0.5 mm. (A, right) Microvessel number and total length of microvessel were quantified. $n=6$ in each group. (B) *In vivo* angiogenesis using a Directed *In Vivo* Angiogenesis Assay kit. (B, left) Photographs of silicone cylinders at 15 days after implantation in male WT and male ARKO mice, the capillary vessels migrated into and proliferated in silicone cylinders with angiogenic factors (FGF+VEGF) from subcutaneous vessels. The value of WT control was set as 1. $n=17$ in each group, 2 cylinders being implanted in each mouse. Arrowed line indicates 1.0 cm. (B, right) Relative invasion to male WT mice without angiogenic factors (PBS) was determined by fluorescence of cell pellets labeled with FITC-Lectin. $*P<0.05$, $**P<0.01$ using Dunn's test following the Kruskal-Wallis test. Bars represent mean values in each group. (C) Bone marrow (BM) transplantation model. Limb survival rate after ischemic surgery in male WT mice receiving male WT BM (WT-BM→WT) or male ARKO BM (KO-BM→WT), and male ARKO mice receiving male WT BM (WT-BM→KO) or male ARKO BM (KO-BM→KO). $n=10$ in each recipient group. $**P<0.01$ vs. male ARKO mice receiving the same genotype of BM at day 21 after ischemia using the log-rank test with Bonferroni-corrected pairwise comparisons. (D) Ratio of blood flow in ischemic to nonischemic limb by LSBF at each time point after

ischemic surgery in male WT and male *ARKO* mice with BM transplantation treatment between BM of male WT mice and BM of male *ARKO* mice. $n=10$ in each recipient group. ** $P<0.01$ vs. male *ARKO* mice receiving the same genotype of BM at days 3 to 21 after ischemia using a linear mixed effects regression analysis. Error bars represent SEM.

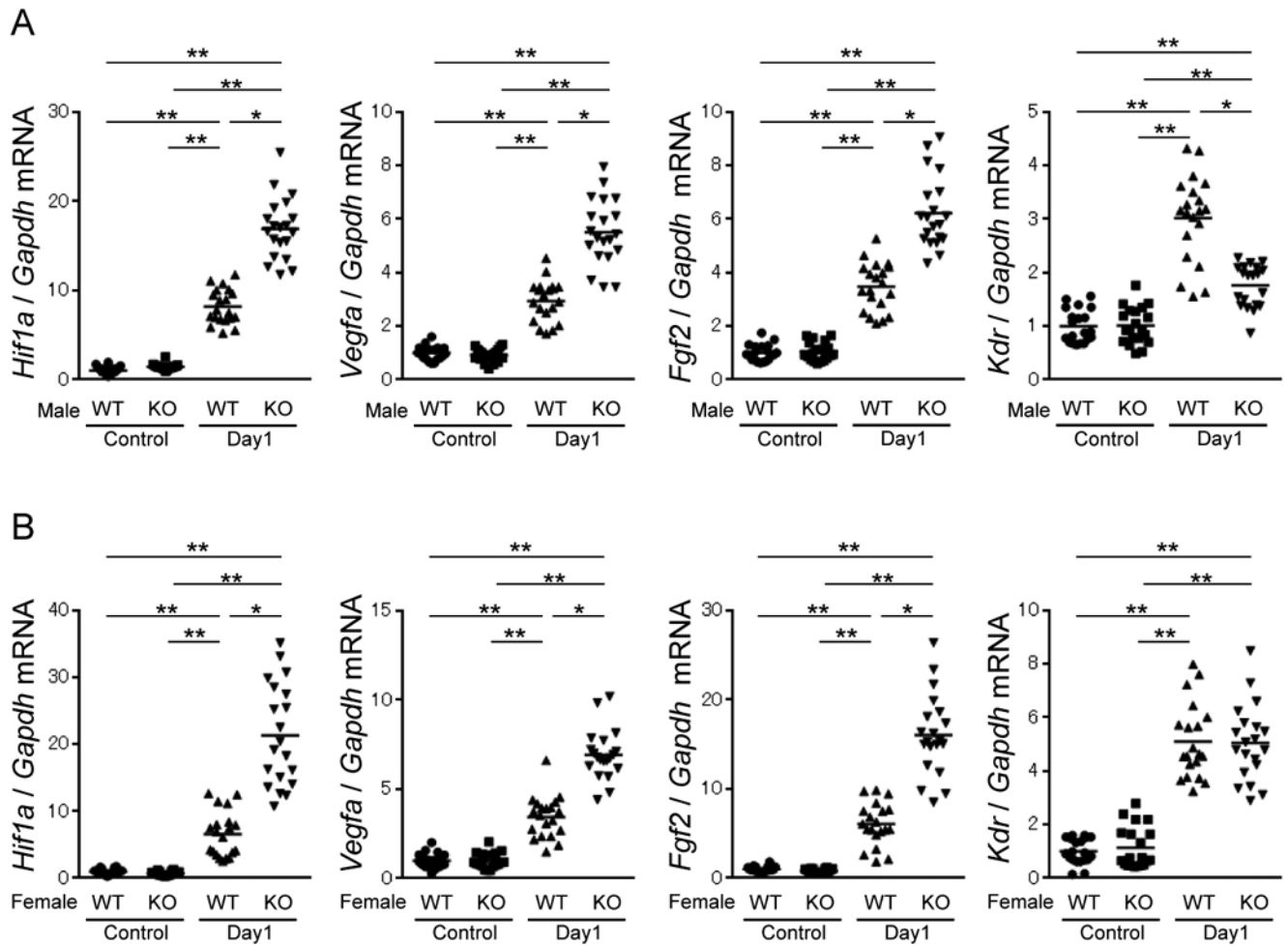


Figure 5. Aberrant expression and activation of angiogenesis related factors in ischemic muscles of male and female ARKO mice

Quantitative PCR analysis for angiogenesis-related factors in skeletal muscles of male WT mice and male ARKO mice (A) or female WT mice and female ARKO mice (B) before surgery (control) and at day 1 after ischemic surgery. mRNA level of *Gapdh* served as an internal control. The value of WT control was set as 1. $n=20$ in each group. * $P<0.05$, ** $P<0.01$ using Dunn's test following the Kruskal-Wallis test. Bars represent mean values in each group.

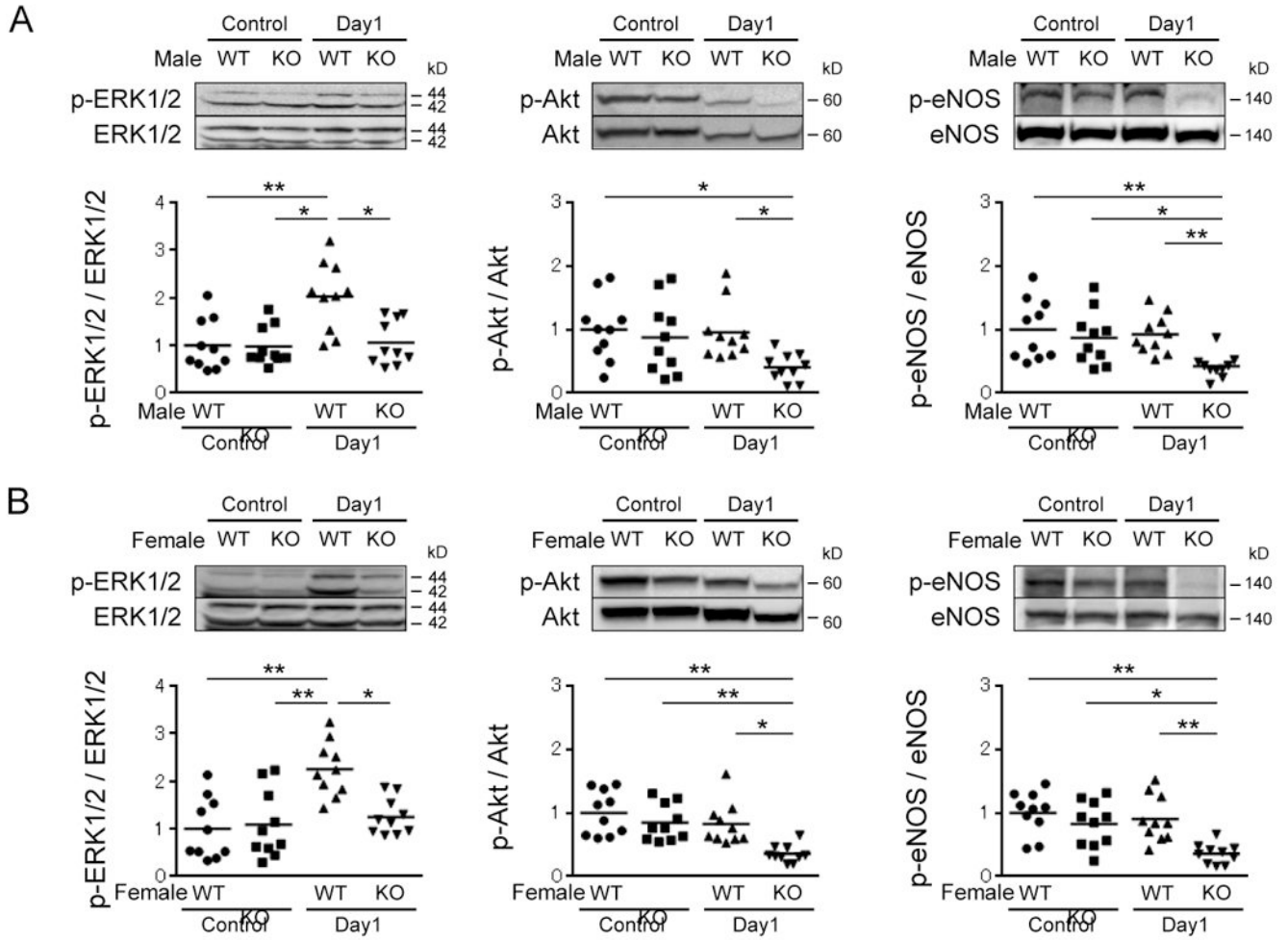


Figure 6. Reduced phosphorylation of factors involved in cell survival and angiogenesis in ischemic muscles of male and female ARKO mice

Western blot analysis of total and phosphorylated ERK1/2, Akt and eNOS in skeletal muscles of male WT mice and male ARKO mice (A) or female WT mice and female ARKO mice (B) before surgery (control) and at day 1. The value of WT control was set as 1. $n=10$ in each group. * $P<0.05$, ** $P<0.01$ using Dunn's test following the Kruskal-Wallis test. Bars represent mean values in each group.

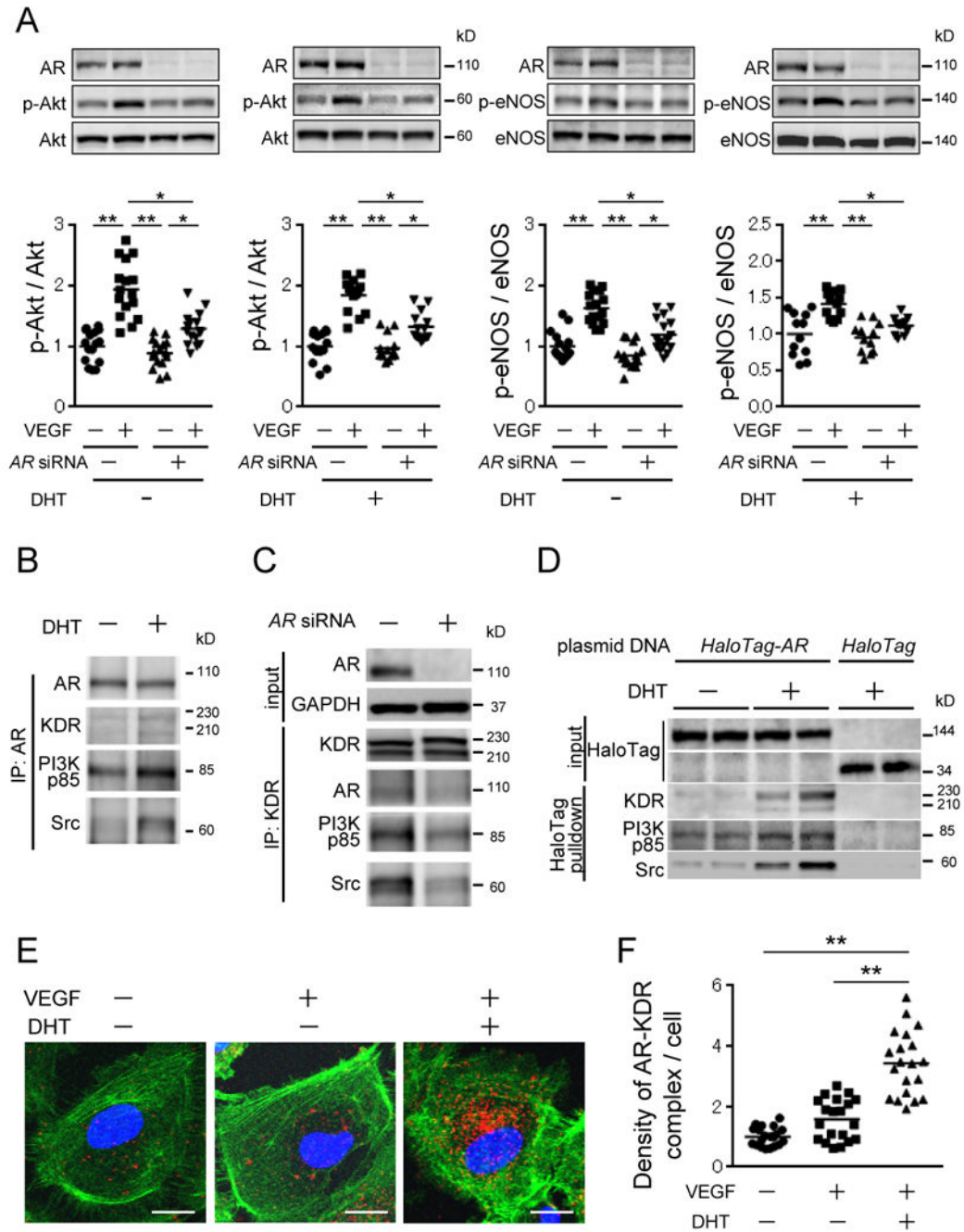


Figure 7. Association between AR and VEGF receptor signaling pathway in HUVECs
 (A) VEGF-stimulated phosphorylation of Akt and eNOS in HUVECs with or without AR knockdown. The value of WT control was set as 1. $n=12-16$ in each experiment. (B) Immunoprecipitation experiments using AR antibody with or without DHT. (C) Immunoprecipitation experiments using KDR antibody with or without AR knockdown. (D) HaloTag pull-down assay in HUVECs expressing HaloTag-AR fusion protein. (E and F) *In site* proximity ligation assay using AR and KDR antibodies in HUVECs with or without ligands. (E) Confocal microscopy images indicate endogenous AR-KDR complexes (red dots), nuclear (blue) and filamentous actin (green). Scale bar indicates 20 μm . (F) Quantified results showing fold increase in fluorescent signal density per cell for each condition. Five

different visual fields were randomly selected for counting red dots in four independent samples of each group, and the values of control cells (VEGF (-) and DHT (-)) were set at 1.0. $**P<0.01$ using Dunn's test following the Kruskal-Wallis test. Bars represent mean values in each group.

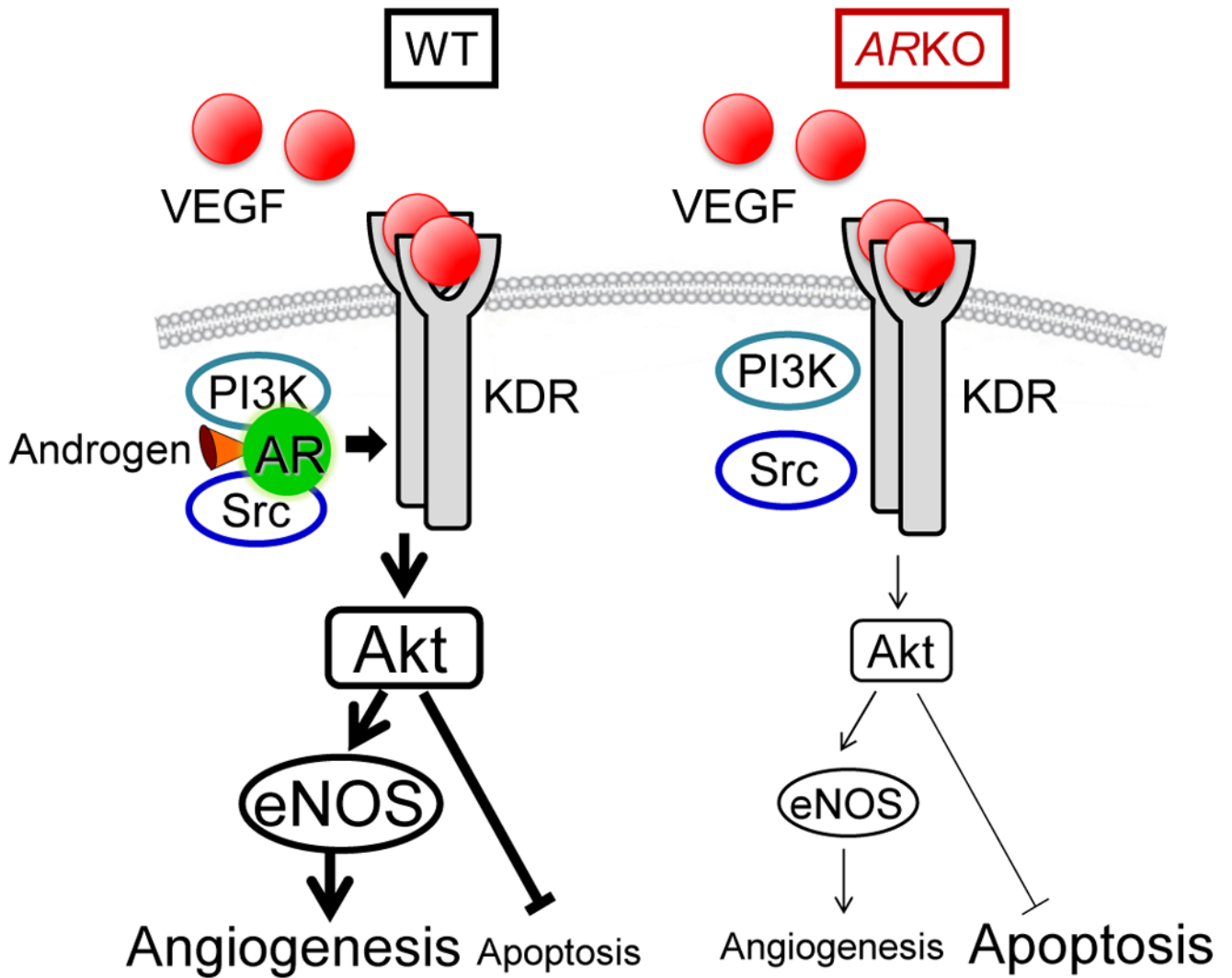


Figure 8. Schematic diagram of associations between AR and VEGF receptor signaling pathways VEGF rapidly induces KDR dimerization and autophosphorylation, followed by the recruitment and activation of Src and PI3K. On the other hand, AR activates the Akt/eNOS cascade depending on association with PI3K and Src. Thus, association between AR and KDR promotes cell survival and angiogenesis via recruitment of Src and PI3K.