Androgen Receptor Promotes Sex-Independent Angiogenesis in Response to Ischemia and Is Required for Activation of Vascular Endothelial Growth Factor Receptor Signaling

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

Methods and Results—Both male and female AR knockout 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 AR knockout mice, reductions in the phosphorylation of the Akt protein kinase and endothelial nitric oxide synthase were observed despite a robust increase in hypoxia-inducible factor 1α 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 endothelial nitric oxide synthase. Immunoprecipitation experiments documented an association between AR and kinase insert domain protein receptor that promoted the recruitment of downstream signaling components.

Conclusions—These results document a physiological role of AR in sex-independent angiogenic potency and provide evidence of novel cross-talk between the androgen/AR signaling and VEGF/kinase insert domain protein receptor signaling pathways. (Circulation. 2013;128:60-71.)

Key Words: Akt1 protein, mouse ■ angiogenesis inducing agents ■ nitric oxide synthase type III receptors, androgen ■ vascular endothelial growth factor receptor-2

Cardiovascular disease (CVD) remains a major cause of human deaths, especially in men, and sex differences in its onset and severity make it a key contributor to the lifespan gap between sexes. Although the sex disparity in CVD has been considered primarily a reflection of estrogen-mediated protection against atherogenesis, conclusive clinical evidence supporting this hypothesis is lacking. In fact, 2 large-scale, prospective, randomized, clinical trials showed no cardiovascular benefits of combined estrogen plus progestin therapy in postmenopausal women. On the other hand, increased understanding of the biological effects of androgens in the cardiovascular system has revealed a close relationship between a decrease in male testosterone levels, called andropause, and an increase in cardiovascular events with aging. Many clinical studies have shown that hypoandrogenemia is associated with CVD, cardiovascular death, metabolic syndrome, visceral fat obesity, type 2 diabetes mellitus, Testosterone replacement therapy has been shown to have beneficial effects on CVD risk factors, including visceral abdominal fat mass, insulin sensitivity, glycemic control, and hyperlipidemia. We have also reported that serum levels of dehydroepiandrosterone sulfate, a major active androgen

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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 system. The biological activities of androgen are mediated predominantly through the transcriptional control of target genes by genomic actions of the nuclear androgen receptor (AR). Androgen also exerts nongenomic actions by interacting with multiple signaling pathways independently of transcriptional control. Such nongenomic effects of androgen may occur through AR in the cytoplasm to induce the rapid activation of kinase signaling cascades.

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 obesity, high-turnover osteopenia, impaired brain masculinization, and aberrant adiponectin expression. In addition, we have demonstrated that AR activation plays a pivotal role in physiological cardiac growth and protection from pathological cardiovascular remodeling induced by angiotensin II and cardiotoxicity induced by the anti-cancer agent doxorubicin.

Although skeletal muscle ischemia caused by peripheral arterial disease and thrombotic disorders is also a critical problem in elderly subjects with andropause, it has been unclear whether AR plays a distinct pathophysiological role in response to skeletal muscle ischemia. 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 novel sex-independent 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 with the Cre-loxP system as previously described. Male ARKO mice exhibited testicular feminization and a reduction in levels of serum gonadal androgens (testosterone and dihydrotestosterone), whereas serum adrenal androgens and estrogen levels remained normal. Female ARKO mice have normal serum hormone levels, including 17β-estradiol, progesterone, testosterone, luteinizing hormone, and follicle-stimulating hormone. 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. We performed the following experimental procedures: macroscopic evaluation of ischemic severity, laser speckle blood flow analysis, immunohistochemistry, real-time polymerase chain reaction analysis, Western blot analysis, aortic ring assay, in vivo angiogenesis assay and bone marrow (BM) transplantation, SiRNA experiments, immunoprecipitation, and in situ proximity ligation assay. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee at the University of Tokushima Graduate School of Health Biosciences. Details of the experimental procedures can be found in the online-only Data Supplement.

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Results

Increased Incidence of Autoamputation in ARKO Mice After Hind-Limb Ischemia

To elucidate the role of AR in ischemic response, we created a model of hind-limb ischemia induced by surgical arterectomy 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 on day 21 after ischemic surgery (Figure 1A and 1B). 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 sexes of WT and ARKO mice by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. The number of TUNEL-positive cells in ischemic skeletal muscle was significantly increased in both male and female ARKO mice compared with the respective male and female WT mice (Figure 2A–2D). 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 polymerase chain reaction 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 ratio of Bcl-2 (Bcl-2) to Bax (BAX) expression than in respective male and female WT mice (Figure 2F–2K). In male mice, the ratio of Bcl-2 to Bax on day 1 decreased on mRNA level, although the protein ratio of those proteins was different result. These results indicate the possibility that there is a sex difference in the stability of Bax and Bcl2 mRNAs. Taken together, these findings indicate that the severity of ischemia-induced cellular apoptosis...
KO mice than in WT mice regardless of sex (Figure 3A, 3B, 3E, and 3F). 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, 3D, 3G, and 3H). In addition, smaller numbers of α-smooth muscle actin–stained cells, indicating vascular pericytes, were observed in male and female ARKO mice after ischemia (Figure I in the online-only Data Supplement).

Attenuated Angiogenic Potency in AR-Deficient Vascular Endothelial Cells
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 4B). Figure 4A shows representative photos and quantitative results of microvascular sprouting on day 7 after aortic ring implantation. We found that the number of sprouting microvessels and the length of microvessels were significantly reduced in aortas from male ARKO mice compared with 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 with male WT mice (Figure 4B). These results are consistent with the notion that AR-deficient vascular endothelial cells have reduced angiogenic potency.

BM Cells Are Not Involved in Impaired Angiogenesis of Male ARKO Mice After Ischemia
BM-derived progenitor cells have been shown to participate in revascularization in ischemic tissue.29 In this regard, it has been proposed that the mobilization of angiogenic progenitors contributes to the effects of androgens on the revascularization process.30 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 4D, 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 a result of 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 polymerase chain reaction analysis. Under nonischemic conditions, there were no significant differences in gene expression levels of proangiogenic factors, including hypoxia inducible factor 1α.
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–endothelial nitric oxide (NO) synthase (eNOS) signaling pathways are activated by the nongenomic actions of AR stimulation. 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 male and female WT mice (Figure 5A and 5B). Because activation of both Akt and ERK1/2 promotes cell survival and because activation of Akt and eNOS enhances angiogenesis, 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 Human Umbilical Vein Endothelial Cells Blunts Activation of the VEGF Receptor Signaling Pathway

To determine whether the 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

(Hif1α), vascular endothelial growth factor A (Vegfa), fibroblast growth factor 2 (Fgfa), and the VEGF receptor Kdr between both sexes of WT and ARKO mice (Figure 5A and 5B). However, after surgery-induced ischemia, expression levels of Hif1α, Vegfa, and Fgfa were augmented prominently in male and female ARKO mice compared with 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).

Figure 2. Accelerated cellular apoptosis in ischemic skeletal muscles of both male and female androgen receptor knockout (ARKO) mice. A and C, Immunofluorescence of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining in ischemic muscle of male wild-type (WT) and ARKO mice (A) or female WT and ARKO mice (C) on day 1 after ischemia. Scale bar, 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 on day 1. Black arrows denote nuclei of skeletal muscle cells; blue arrows, nuclei of endothelial cells. Scale bar, 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 before surgery (control) and on 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 polymerase chain reaction in ischemic muscle of both sexes of WT and ARKO mice before surgery (control) and on day 1 after ischemia. n=16 in each group in G, H, and J; n=12 in each group in K. Bars represent mean values in each group. *P<0.05, **P<0.01 with the Bonferroni-corrected Mann–Whitney U test following the Kruskal–Wallis test.
with or without siRNA-mediated AR knockdown. (In this study, the siRNA reduced AR mRNA levels to 17.0±1.8% of the control and reduced AR protein levels to 20.8±2.0% of the control.) VEGF stimulation in the presence of 5α-dihydrotestosterone (DHT) enhanced Akt and eNOS phosphorylation in control human umbilical vein endothelial cell cultures. In contrast, VEGF-stimulated Akt and eNOS phosphorylation was blunted in human umbilical vein endothelial cells 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 Kinase Insert Domain Protein Receptor, Src, and Phosphoinositide-3-Kinase**

Stimulation by VEGFs rapidly induces kinase insert domain protein receptor (KDR) dimerization and autophosphorylation, followed by recruitment and activation of Src and phosphoinositide-3-kinase (PI3K). AR is also shown to recruit Src, to activate the mitogen-activated protein kinase pathway, and to activate the PI3K-Akt cascade, leading to cell survival and proliferation. Because 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 human umbilical vein endothelial cell lysates with 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). To clarify the association between
KDR and AR, we used HaloTag technology. Exogenous AR-HaloTag fusion proteins and HaloTag control proteins were expressed in endothelial cells; 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, whereas 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

To visualize the association between AR and KDR in vascular endothelial cells, we used an in situ proximity ligation assay, a method used for detecting and imaging protein associations in both tissue sections and cultured cells in vitro. The in situ proximity ligation assay revealed modest AR/KDR proximity signal in human umbilical vein endothelial cells under baseline conditions (Figure 7E, left). VEGF stimulation alone led to a slight increase in the AR/KDR proximity signal (Figure 7E, middle). However, the combination of VEGF and DHT led to markedly greater AR/KDR complex formation (Figure 7E, right, and Figure 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 ischemia-induced cellular
apoptosis of myofibers and endothelial cells. Our study also documents a hitherto unrecognized cross-talk mechanism between the androgen/AR and VEGF/KDR signaling pathways. Collectively, these data provide a molecular rationale for why individuals experiencing andropause are at greater risk for ischemic disease.

Pathophysiological roles of AR in females are largely unknown. Shiina et al have demonstrated that 8-week-old female ARKO mice manifest reduced ovarian follicle numbers, impaired mammary development, and reduced numbers of pups. In addition, they have shown that 40-week-old ARKO mice have no ovarian follicles, leading to infertility with aberrant gene expression levels of folliculogenesis-related factors. These results and our present study suggest that liganded or unliganded AR activation might partially participate in biological homeostasis of multiple organs dependently or independently of sex and estrogen.

Aspects of our study are consistent with a prior report by Sieveking et al showing that androgens have a positive effect on angiogenesis. For example, they reported that castration led to a reduction in ischemia-induced angiogenesis. However, use of the ARKO mouse in the present 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 ischemia. 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 (AR KO mice) or absence (ovariectomized WT mice) of estrogen in female mice, which can influence a revascularization response. Alternatively, the presence (ovariectomized WT mice) or absence (AR KO 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 actions. We speculate that unliganded AR also plays a role in angiogenesis after ischemia regardless of sex. Further experiments are required to clarify these issues.

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

**Figure 5.** Aberrant expression and activation of angiogenesis-related factors in ischemic muscles of male and female androgen receptor knockout (ARKO) mice. Quantitative polymerase chain reaction analysis for angiogenesis-related factors in skeletal muscles of male and female wild-type (WT) mice and male ARKO mice (A) or female WT mice and female ARKO mice (B) before surgery (control) and on day 1 after ischemic surgery. The mRNA level of Gapdh served as an internal control. The value of WT control was set as 1. n=20 in each group. Bars represent mean values in each group. **P<0.05, ***P<0.01 with the Dunn test following the Kruskal–Wallis test.
AR deficiency led to molecular changes associated with diminished cell viability in the ischemic limb, including a reduction in the ratio of Bcl-2 to Bax 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 the determination of the apoptotic threshold. Lin et al reported that AR is able to counteract BAX-mediated apoptosis in the prostate gland in an androgen-dependent and -independent manner. 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 mice. 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 genes. Additionally, Akt has been shown to be a cytoplasmic serine/threonine kinase that contributes to the control of apoptosis machinery and cellular metabolism. 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. 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 phosphorylation. Consistent with these observations, the present study demonstrated that AR deficiency accelerates cellular apoptosis through a reduced Bcl-2–to–BAX ratio and impaired phosphorylation of ERK1/2 and Akt under ischemic stress regardless of sex.

The PI3K/Akt pathway is activated by VEGF and is capable of modulating cell survival, migration, tube formation, and NO release. 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. It is well known that Akt activates eNOS and enhances NO production and that release of NO from vascular endothelial cells is essential for promoting angiogenesis and collateral vessel remodeling in response to ischemia. 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 excess. 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 rapid elevations 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 exhibit marked edema and a dusky red–colored skin appearance on day 1 after surgery is consistent with exaggerated ischemic stress. Previous studies have shown that castration rapidly reduces blood flow to the rat ventral prostate gland with acceleration of epithelial cellular apoptosis and tissue hypoxia, and we have reported that AR deficiency reduces NO bioavailability. Thus, the lack of androgen action may lead to prominent tissue hypoxia resulting from acute circulatory dysfunction. 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 compared with WT mice.

Interestingly, the enhanced gene expression levels of Kdr as a compensatory response against ischemic stress were 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 uses its receptor KDR, also known as VEGFR-2 or fetal liver kinase-1, to induce angiogenic responses mediated by subsequent activation of signaling cascades such as PI3K-Akt and mitogen-activated protein kinase. 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 cells. Their results and our present observations support the notion that AR activation is required for compensatory upregulation of Kdr gene expression in response to ischemia, at least in males. Although the relationships between sex hormones, AR, and Kdr gene expression in female mice remain 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 upregulate the 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. Although their experiments were performed using cultured endothelial cells, 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 attributable to differences in the experimental systems in the 2 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 are 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 mitogen-activated protein kinase. 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.

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Disclosures
None.

References

Figure 8. Schematic diagram of the associations between androgen receptor (AR) and vascular endothelial growth factor (VEGF) receptor signaling pathways. VEGF rapidly induces kinase insert domain protein receptor (KDR) dimerization and autophosphorylation, followed by the recruitment and activation of Src and phosphoinositide-3-kinase (PI3K). On the other hand, AR activates the Akt/endothelial nitric oxide synthase (eNOS) cascade, depending on the association with PI3K and Src. Thus, the association between AR and KDR promotes cell survival and angiogenesis via recruitment of Src and PI3K.


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**CLINICAL PERSPECTIVE**

In addition to menopause, andropause has been well recognized as a risk factor for cardiovascular diseases. Therefore, sex-specific clinical and experimental data are important in delineating sex-dependent differences in the incidence of and vulnerability to cardiovascular diseases. Here, we show that androgen receptor activation plays an important role in angiogenesis after ischemia regardless of sex. This study identifies the function of the androgen receptor in the compensatory response to ischemia in vivo and defines its mechanism of action as the association of androgen receptor with a vascular endothelial growth factor receptor and activation of proangiogenic 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 have been under clinical development, and trials are underway in patients with sarcopenia and cachexia, conditions that are closely associated with cardiovascular complications. On the basis of the present study, we speculate that the appropriately targeted regulation of androgen receptor function in ischemic tissues may lead to novel therapeutic approaches for the treatment of cardiovascular diseases in both sexes.
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SUPPLEMENTAL MATERIAL

Murine hind limb ischemia model

All surgical procedures and laser speckle blood flow analysis (see below) were performed under anesthesia with intraperitoneal pentobarbital sodium salt injection (80 mg/kg). To induce unilateral hind limb ischemia, skin incision was performed at the mid-portion of the right hind limb overlying the femoral vessels. The femoral vessels were then gently isolated without damaging the femoral nerve. The proximal portion of the femoral artery and the distal portion of the saphenous artery were ligated with 7-0 silk ligatures (Natsume Seisakusyo Co., Ltd., Tokyo, Japan). The remaining branches between these two sites as well as veins were all dissected free and then excised. The overlying skin was closed using a 3-0 silk suture (Akiyama Seisakusyo, Tokyo, Japan). After surgery, mice were kept on a heating plate at 37°C to monitor the operated animals until they recovered completely from anesthesia. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, The University of Tokushima Graduate School.

Macroscopic evaluation of ischemic severity

After the operation, clinical outcomes of all mice (n=17 in each male mouse group, n=25 in the female WT mouse group and n=12 in the female ARKO mouse group) were observed and recorded at 4 time points (at days 3, 7, 14 and 21 after surgery). The ischemic limb was macroscopically evaluated, and diagnosis of hind limb autoamputation was determined as extension of necrosis above the crus (knee loss and total hind limb loss).
**Laser speckle blood flow analysis**

We measured hind limb blood flow using a laser speckle blood flow (LSBF) imager (OMEGAZONE OZ-1, OMEGAWAVE, Inc., Tokyo, Japan) at 6 time points (before surgery, and at days 1, 3, 7, 14 and 21 after surgery). In the LSBF imager, low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as yellow to red. The stored perfusion values behind the color-coded pixels remain available for data analysis. Previous studies \(^1,^3\) established that laser Doppler flow velocity correlates with capillary density in ischemic limbs of animal models, and Briers et al. demonstrated that the two techniques of laser Doppler blood flow analysis and LSBF analysis were essential for measuring line-of-sight velocities \(^4\). Excess hair was removed from the hind limb using a depilatory cream, and before initiating scanning, mice were placed on a heating plate at 37°C to minimize variations in temperature. For each time point, we obtained two consecutive LSBF images over the same region of interest (legs and feet) (\(n=17\) in male WT mouse group and \(n=8\) in male ARKO mouse, \(n=16\) in female WT mouse group and \(n=7\) in female ARKO mouse group). Accordingly, the averaged flows of the ischemic and non-ischemic limbs were calculated on the basis of colored histogram pixels. To avoid the influence of ambient light and temperature, LSBF data were expressed as the ratio of ischemic (right) to normal (left) hind limb perfusion. At day 21, the male and female mice were sacrificed, and the thigh adductor muscles of bilateral limbs were harvested for capillary density analysis.

**TUNEL staining**

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<Immunofluorescence> At day 1 after hind limb ischemic surgery, the male and female mice ($n=8$ in each male mouse group) were euthanized. After the thigh adductor muscles of ischemic limbs had been removed, they were embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and snap-frozen in liquid $N_2$. Multiple cross cryosections of 6 $\mu$m in thickness were prepared, and apoptotic cells were analyzed by TUNEL staining using an In situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). Briefly, the samples were fixed in 4% paraformaldehyde for 20 min at room temperature and then washed with PBS for 30 min. After incubating in permeabilization solution for 2 min and washing with PBS two times, the samples were incubated with TUNEL reaction mixture at 37$^\circ$C in a humid condition for 1 hr and then encapsulated by mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA). We evaluated the number of apoptotic cells using fluorescence microscopy (Nikon Corporation, Tokyo, Japan). TUNEL-positive cells were counted in five randomly selected microscopic fields in each plate (magnification, 100×), and the percentages of apoptotic cells were expressed as TUNEL-positive cells per total number of DAPI-positive cells.

<Enzyme antibody technique> The ischemic adductor muscles at day 1 were fixed in 4% paraformaldehyde overnight. After fixation, the muscles were embedded in paraffin and were serially cut into 3-$\mu$m-thick slices. After being deparaffinized and hydrated, the sections were washed in deionized water, and endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Next, the sections were treated with 20 $\mu$g/ml proteinase K for 10 min at room temperature and rinsed in PBS. Then the sections were incubated with a reaction reagent (ApopTag Peroxidase In Situ Apoptosis Detection Kit, CHEMICON International, Inc.,
Temecula, CA) for 60 min at 37°C and with 1:500-diluted peroxidase-conjugated streptavidin for 30 min at room temperature, rinsed in PBS, incubated with 3.3’-diaminobenzidine tetrahydrochloride for 10 min at room temperature, washed in water, and finally counterstained with methyl green for 30 min. The sections were examined under a light microscope (Olympus Corporation, Tokyo, Japan, magnification, 400×).

**CD31 and αSMA immunostaining**

As described above, fresh-frozen sections of hindlimb adductor muscles at day 21 after ischemic surgery (n=16 in group) were prepared. The sections were histochemically stained with CD31 antibody (BD Pharmingen, Franklin Lakes, NJ) to identify capillary endothelial cells and smooth muscle actin (SMA) antibody (Dako North America, Inc. Carpinteria, CA) to identify vascular pericytes. Immunostains were visualized by using an amino acid-polymer system (Nichirei Bio, Tokyo, Japan), DAB (for CD31) and Permanent Red (for SMA). Capillaries and arterioles in both ischemic and nonischemic limbs were analyzed for specific evidence of neovascularity. Endothelial cells positively stained with CD31 antibody, vascular pericytes positively stained with SMA antibody and muscle fibers were counted under a light microscope (Olympus Corporation, Tokyo, Japan, magnification, 200×). Five random microscopic fields from 2 different sections (50 μm apart) in each mouse were counted. Capillary density was expressed as number of CD31-positive cells per muscle fiber or per square millimeter. Arteriolar (pericyte) numbers were expressed as number of SMA-positive cells per square millimeter \(^2\).
**Aortic ring assay**

Descending thoracic aortas from male WT and ARKO mice were excised (n=6 in each male mice group), and fibro-adipose tissue around the aorta was carefully removed. Under a dissecting microscope, five 1-mm-thick aortic rings were prepared from each aorta. The aortic rings were embedded in 3-dimensional gels of Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA). Matrigel Basement Membrane Matrix solution (200 μl) was transferred to each well of 48-well plates and gelatinized to make a base layer at 37°C for 30 min. Each aortic ring was placed on the basal layer of the Matrigel in the center of the well, covered with an additional 100-μl aliquot of Matrigel solution and allowed to gel for 30 min. Five hundred μl of EGM-2 medium (Lonza, Walkersville, MD) was then added to each well and cultured for 7 days. At day 7, the total number and total length of sprouting microvessels were determined under a light microscope (Olympus Corporation, Tokyo, Japan, magnification, 40×) and using ImageJ version 1.29 6,7.

**In vivo angiogenesis assay**

Quantitative *in vivo* angiogenesis assay using DIVVA (Trevigen, Inc. Gaithersburg, MD) was performed in male WT and ARKO mice. According to the manufacturer’s instructions, implant-grade silicone cylinders were each filled with 20 μl of basement membrane extract (BME) premixed with PBS as negative control or 30 ng FGF-2 with 10 ng VEGF. These cylinders were implanted subcutaneously in the dorsal flanks of male WT and ARKO mice (n=17 for each group, 2 cylinders implanted in each mouse). At 15 days after implantation, vascular endothelial cells had migrated into and proliferated in the BME with the angiogenic
factor to form vessels in the silicone cylinders. The cylinders were removed from each mouse
and BME/vessel complex was extracted and collected as cell pellets. The cell pellets were
labeled with DIVVA FITC-Lectin and fluorescence was measured.

**Bone marrow transplantation (BMT)**

Nineteen-week-old male WT and ARKO mice (n=10 in each recipient group) were irradiated
with a dose of 9.5 Gy from an X-ray source at a dose rate of approximately 0.90 Gy/min. Bone
marrow cells (BMCs) from age-matched donor male WT and ARKO mice were harvested by
flushing the femurs and tibias, and red cells were removed by lysis using ammonium chloride.
BMCs in suspension were counted, and 1×10^7 cells/mouse were injected through the orbital
vein of the recipient immediately after irradiation. In order to determine whether BMT was
successfully completed, we created chimeric mice transplanted with BMCs from CD45.1 mice
(B6.SJL-PtprcalPep3b/BoyJ). The chimeric ratio of BMCs was more than 95% as determined
by fluorescence-activated cell sorting analysis of the chimeric mice at 6 weeks after BMT (data not shown). Six weeks after BMT, the recipient male WT and ARKO mice were
subjected to hindlimb ischemia as described above. At indicated time points, we also
examined limb survival rate and blood flow reperfusion.

**Western blot analysis**

For Western blotting analysis and real-time PCR analysis (see below), adductor muscles before
ischemic surgery and ischemic muscles at day 1 after surgery were excised (n=10 in each group).
Phosphorylation of Akt, eNOS and Erk1/2 and expression of Bcl-2 and BAX were evaluated by
Western blot analysis. Protein extraction from muscle and Western blot analysis were performed as described previously. In brief, 50-μg protein extracts from the muscles of WT and ARKO mice were boiled for 5 min in Laemmli sample buffer and then run on SDS-PAGE. The protein extracts were then transferred to a PVDF membrane (Millipore Corporation, Bedford, MA). The membrane was blocked for 20 min at room temperature with SuperBlock T20 TBS Blocking Buffer (Thermo Scientific, Rockford, IL). The blots were incubated overnight at 4°C with each primary antibody, followed by incubation for 1 hr with anti-rabbit secondary antibody (horseradish peroxidase-conjugate). Immunoreactive bands were visualized using enhanced chemiluminescence with ECL-PLUS reagents (GE Healthcare, Buckinghamshire, UK) and exposure to a lumino image analyzer (LAS-3000mini) (Fujifilm Corporation, Tokyo, Japan). The signals were quantified by densitometry using ImageJ version 1.29. We analyzed 8 independent samples for each time point in each group and detected the phosphor-specific antibody first, then the total antibody, and finally the internal control antibody. Phospho-specific proteins were normalized by total protein, and total protein was corrected by GAPDH as an internal control. We used primary antibodies against phosphorylated Akt (Ser473), total Akt, phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, phosphorylated eNOS (Ser1177), GAPDH (Cell Signaling Technology, Beverly, MA), total eNOS (BD Bioscience, San Jose, CA), Bcl-2 and BAX (Santa Cruz Biotechnology, Inc., CA).

Quantitative real-time PCR

RNA extraction and reverse transcriptase-polymerase chain reaction were performed as described previously. In brief, thigh adductor muscles were homogenized in TRIzol
(Invitrogen, Carlsbad, CA) and total RNA was extracted. Total RNA of 1 μg was used for cDNA synthesis with a QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. The PCR mixture contained cDNA, generated from 2.5 ng of total RNA, 0.1 nmol/l forward and reverse primer mix, and SYBR Green (Platinum SYBR Green qPCR SuperMIX-UDG, Invitrogen Carlsbad, CA). Assays were performed with a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification included one stage of 2 min at 50°C and one stage of 2 min at 95°C followed by 40 cycles of a 2-step loop: 15 seconds at 95°C and 30 seconds at 60°C. Commercially available PCR primers were purchased from Perfect real-time primer (TAKARA BIO INC. Ohtsu, Japan) for Hif1a, Vegfa, Fgf2, Kdr, Bcl2, Bax and Gapdh. Results were analyzed with the 7300 System software, and transcript levels were adjusted relative to the expression of Gapdh as an internal control. The twenty independent samples for each time point in each group were used, and duplicate results per sample were averaged.

**Cell preparation**

Male HUVECs were purchased from Cell Applications Inc. (San Diego, CA) and were maintained in EGM-2 medium (Lonza, Walkersville, MD) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells were grown to confluence and then the medium was changed without a growth factor (EBM-2 medium with 0.5% FBS) and cultured overnight. Cells were treated with or without 100 nM 5alpha-dihydrotestosterone (DHT) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and 10 ng/ml human recombinant VEGF (Sigma-Aldrich, Inc., Saint Louis, MO) for 15 min. Cultured cells with various treatments were washed twice with cold
PBS and harvested after being lysed for 30 min at 4°C in a lysis buffer (Cell Signaling Technology, Beverly, MA). Supernatants obtained by centrifugation were used for Western blot analysis and immunoprecipitation. The procedure for Western blot analysis was described previously 9,12. Antibodies against phosphorylated Akt (Ser473), total Akt, phosphorylated eNOS (Ser1177) and GAPDH were purchased from Cell Signaling Technology (Beverly, MA). Total eNOS was purchased from BD Bioscience (San Jose, CA).

**Small interfering RNA (siRNA) experiments**

HUVECs were plated in 12-well plates (1×10^5 per well) in EGM-2 medium without antibiotics. Twenty-four hours later, cells were transfected with siRNA targeting AR and nontargeting siRNA (negative control) using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA). The siRNA of AR (Stealth RNAi siRNA; Invitrogen, Carlsbad, CA) and negative control duplexes (Stealth Select RNAi; Invitrogen, Carlsbad, CA) were purchased. According to the manufacturer’s protocol, 2 μl of lipofectamine RNAiMAX and 2 pmol RNAi duplex were added directly to 200 μl of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA) per well and incubated for 15 min. The RNAi duplex-Lipofectamine RNAiMAX complexes were added to each well containing cells, and the cells were incubated. The medium was changed without a growth factor (EBM-2 medium with 0.5% FBS) at 24 hr after transfection, and the transfected cells were stimulated with 10 ng/ml VEGF and 100 nM DHT for 15 min at 36 hr after transfection. Then the cells were washed with PBS and proteins were isolated from HUVECs. At 36 hr after transfection with this siRNA, mRNA and protein expression levels of AR were reduced to 13% and 15% of the control levels, respectively (data not shown).
**Immunoprecipitation**

The lysates of treated HUVECs, including 500 μg protein, were precleaned with protein A/G agarose beads (Santa Cruz Biotechnology, Inc., CA) and rabbit IgG antibody (Cell Signaling Technology, Beverly, MA) at 4°C for 1 hr, and then supernatants were collected. The lysates were incubated with the first protein-specific antiserum (anti-AR antibody (Santa Cruz Biotechnology, Inc., CA) and anti-KDR (VEGFR2) antibody (Cell Signaling Technology, Beverly, MA)) at 4°C overnight and then gently agitated with agarose beads for 1 hr. Immune complexes were collected by centrifugation followed by 3 washes with lysis buffer and boiling in sample buffer for 5 min. The immunoprecipitated proteins were subjected to Western blot analysis. Antibodies against anti-Src and PI3kinase p85 were purchased from Cell Signaling Technology (Beverly, MA).

**HaloTag pull-down assay**

HUVECs were grown to 80% confluency and transfected with plasmid DNA encoding HaloTag plus human AR cDNA (Kazusa DNA Res. Inst., Chiba, Japan) and control HaloTag protein (Promega Corporation, Madison, WI) using Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. At 24 hr after transfection, the transfected cells in EGM-2 medium including VEGF were stimulated with 100 nM DHT for 15 min and harvested. Cell lysates including HaloTag-AR fusion protein and control lysates were prepared using dedicated lysis buffer and protease inhibitor cocktail (Promega Corporation), HaloTag Pull-down assay was performed with HaloLink magnetic beads (Promega Corporation).
according to the manufacturer’s protocol. Cell lysates were incubated with prepared HaloLink magnetic beads for overnight at 4°C, washed 4 times and resuspended in SDS gel loading buffer. Samples were heated to 95°C for 5 min, remove supernatant and load on an SDS-PAGE gel and analyzed by Western blot using anti-KDR (VEGFR2), Src and PI3 kinase p85 antibody (Cell Signaling Technology) and anti-HaloTag antibody (Promega Corporation).

In situ proximity ligation (in situ PLA) assay

HUVECs grown on a culture slide (BD Biosciences, Bedford, MA) were starved without a growth factor overnight and incubated in the presence or absence of 10 ng/ml VEGF and 100 nM DHT for 10 min. The cells were washed with chilled PBS and fixed with 4% paraformaldehyde for 30 min, permeabilized by 0.5% Triton X-100, and thereafter subjected to in situ PLA using Duolink II PLA probe anti-Rabbit PLUS, anti-Mouse MINUS and Detection Reagents (Olink Bioscience, Uppsala, Sweden) according to the manufacturer’s instructions. Briefly, slides were blocked and incubated overnight with primary antibodies from two different species directed against mouse anti-KDR (VEGFR2) (Cell Signaling Technology) and rabbit anti-AR (Abnova Corporation, Taipei, Taiwan). The slides were then incubated with secondary antibodies (anti-mouse and anti-rabbit) conjugated with PLA probes PLUS and MINUS, and the two oligonucleotides and ligase were added. The oligonucleotides would hybridize to the two PLA probes and join to a closed circle if they were in close proximity. Nucleotides and fluorescently labeled oligonucleotides were added together with polymerase, and the PLA signals were visible as a distinct fluorescent spot. Next, filamentous actin staining was performed. Fluorescent phallotoxin staining solution (Alexa Fluor 488 phalloidin,
Molecular Probes, Inc., Eugene, OR) was placed on the slide for 20 min at 37°C and washed two times with PBS. The cells were air-dried and then mounted in mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA) and analyzed in a confocal microscope (Nikon Corporation, Tokyo, Japan). We chose randomly five microscopic fields in four independent preparations individually and evaluated the density of PLA signals per cell (magnification, 400×) \(^\text{13}\).
Supplemental References


Figure legend

Supplemental Figure 1.

Reduced arteriolar numbers in both male and female ARKO mice after ischemia

(A and C) α-SMA immunohistochemical staining to determine arterioles in ischemic and nonischemic thigh adductor muscles in WT and ARKO mice at day 21 after surgery (A: male mice, C: female mice). Scale bar indicates 100 μm. (B and D) Quantification of arteriolar numbers is expressed as αSMA-positive cell number per square millimeter in WT and ARKO mice (B: male mice, D: female mice). n=16 in each group. **P<0.01 using Dunn’s test following the Kruskal-Wallis test. Bars represent mean values in each group.
**Supplemental Figure 1**

**A**

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**B**

![Graph showing αSMA number / mm^2 for Male WT and KO](image)

**C**

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**D**

![Graph showing αSMA number / mm^2 for Female WT and KO](image)