Androgen Receptor Gene Knockout Male Mice Exhibit Impaired Cardiac Growth and Exacerbation of Angiotensin II-induced Cardiac Fibrosis

Androgen has anabolic effects on cardiac myocytes and has been shown to enhance left ventricular enlargement and function. However, the physiological and pathophysiological roles of androgen in cardiac growth and cardiac stress-induced remodeling remains unclear. We aimed to clarify whether the androgen-nuclear androgen receptor (AR) system contributes to the cardiac growth and angiotensin II (Ang II)-stimulated cardiac remodeling by using systemic AR-null male mice. AR knock-out (ARKO) male mice, at 25 weeks of age, and age-matched wild-type (WT) male mice were treated with or without Ang II stimulation (2.0 mg/kg/day) for 2 weeks. ARKO mice with or without Ang II stimulation showed a significant reduction in the heart-to-body weight ratio compared with those of WT mice. In addition, echocardiographic analysis demonstrated impairments of both the concentric hypertrophic response and left ventricular function in Ang II-stimulated ARKO mice. Western blot analysis of the myocardi um revealed that activation of extracellular signal-regulated kinases (ERK) 1/2 and ERK5 by Ang II stimulation were lower in ARKO mice than those of WT mice. Ang II stimulation caused more prominent cardiac fibrosis in ARKO mice than in WT mice with enhanced expression of types I and III collagen and transforming growth factor-β1 genes and with increased Smad2 activation. These results suggest that, in male mice, the androgen-AR system participates in normal cardiac growth and modulates cardiac adaptive hypertrophy and fibrosis during the process of cardiac remodeling under hypertrophic stress.

Androgen exerts a variety of biological effects in many target organs, including male genitalia, brain, and skeletal tissues (1, 2). Most of these actions are mediated through the transcriptional control of particular sets of target genes by the nuclear androgen receptor (AR). AR is a ligand-inducible transcription factor that belongs to the nuclear receptor superfamily (3, 4). Upon hormone binding, AR is translocated from the cytosol into the nucleus where it binds specific promoter elements. A number of coregulators and/or coregulator complexes are then recruited to AR, which then activates or represses the transcription of various target genes (2, 3, 5–7). To clarify the physiological function of androgen via AR transcriptional regulation, we generated AR knock-out (ARKO) mice by means of the Cre-loxP system and have reported that ARKO male mice manifest late-onset obesity (8), high turnover osteopenia (9), and impaired brain masculinization (10).

In addition to the classic target tissues of androgens, the AR gene is also expressed in mammalian cardiomyocytes, suggesting that androgens may play a role in the heart (11). In fact, Marsh and colleagues (11) have shown that androgens produce cardiac hypertrophy by a direct, receptor-specific mechanism. They also revealed that androgens regulate functional expression of an L-type calcium channel in isolated rat ventricular myocytes, leading to a modulation of cardiac performance in males (12). Li et al. (13) reported that either castration or flutamide, an AR antagonist, markedly attenuated cardiac hypertrophy and fibrosis in guanylyl cyclase-A knock-out male mice. These previous studies have helped in delineating the influence of the androgen-AR system in the heart. However, to prove the physiological and pathophysiological roles of the androgen-AR system on cardiac structure and function, in vitro experiments as well as in vivo studies using pharmacological manipulations have limitations.

In the present study, to define the biological significance of cardiac AR function, we carried out studies using ARKO male mice. We obtained in vivo evidence that the androgen-AR system plays a pivotal role in normal cardiac growth and in cardiac protection against angiotensin II (Ang II)-induced cardiac fibrosis in male mice.

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The abbreviations used are: AR, nuclear androgen receptor; ARKO, AR knockout; WT, wild-type; Ang II, angiotensin II; HW, heart weight; BW, body weight; CSa, cross-sectional area; ERK, extracellular signal-regulated kinase; ANP, A-type natriuretic peptide; BNP, B-type natriuretic peptide; αMHC, α-myosin heavy chain; βMHC, β-myosin heavy chain; AT1AR, angiotensin II type 1a receptor; AT2R, angiotensin II type 2 receptor; TGF-β1, transforming growth factor β1; MAP, mitogen-activated protein; LV, left ventricular; LVDd, left ventricular diastolic dimension; LVM, left ventricular mass; RWT, relative wall thickness; FS, fractional shortening.
**ABERRANT CARDIAC GROWTH AND FIBROSIS IN ARKO MALE MICE**

**EXPERIMENTAL PROCEDURES**

**Animal Preparation—ARKO male mice were generated by targeted disruption of the AR gene using the Cre-loxP system. ARKO male mice with C57BL6/CBA hybrid backgrounds were generated and maintained as previously reported (8–10). In ARKO male mice, testicular androgen production was severely impaired, leading to a reduction in serum gonadal androgen levels, whereas serum adrenal androgen and estrogen levels remained normal (9). We used littermate wild-type (WT) male mice and ARKO male mice in this study and divided these mice into four groups; WT male mice with or without Ang II stimulation and ARKO male mice with or without Ang II stimulation. Ang II (WAKO, Japan) dissolved in saline was continuously and subcutaneously infused at a rate of 2.5 μg/kg per min for 5 weeks using an osmotic minipump (Alza Corp., Mountain View, CA). Experiments were conducted in these male mice at 25 weeks of age. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, The University of Tokushima Graduate School.**

**Measurements of Blood Pressure and Heart Rate—Systolic blood pressure and heart rate were measured using a noninvasive computerized tail-cuff system (BP98A Sofrion Corp., Tokyo, Japan) at 2 weeks after pump implantation. Unanesthetized mice from each group were anaesthetized with isoflurane in N2O and placed in a holding device mounted on a thermostatically controlled warming plate, maintained at 37 °C. Systolic blood pressure and heart rate were measured on two consecutive days, and at least 10 readings were taken for each measurement.**

**Echocardiographic Analysis—Transthoracic echocardiography was performed using a 15-MHz imaging transducer (Aplo 80 Toshiba Medical System, Tokyo, Japan). Mice from each group were anesthetized with pentobarbital sodium (5 mg/kg), and transversal sections were scanned by intraperitoneal injection with 20 mg/kg of 2.5% pentobarbital. The left hemithorax of each mouse was carefully shaved, and M-mode images of the left ventricle were recorded. Left ventricular anterior wall thickness (LAV), left ventricular end-diastolic dimension (LVdD), left ventricular end-systolic dimension (LVDs), and left ventricular posterior wall thickness (PW) were measured. All measurements were performed using the leading edge-to-leading edge convention adopted by the American Society of Echocardiography. Percent fractional shortening (FS), left ventricular mass (LVM), and relative wall thickness (RWT) were calculated as follows: FS = [(LVdD − LVDs)/LVdD] × 100, LVM = 1.05 × ([LVDd + AW + PW]/1000 − LVDs/1000 + 0.6) and RWT = (AW + PW)/LVdD.

**Determination of Heart-to-Body Weight Ratio and Histological Analysis—At the end of pump infusion, mice from each group were anaesthetized, and the hearts were excised. The weight of the whole heart (HW) was measured, and the ratio of HW to body weight (HW/BW) was estimated, and the hearts were excised. The weight of the whole heart (HW) was measured, and the ratio of HW to body weight (HW/BW) was estimated after correction for loading differences by measuring the amount of 28 S rRNA. The intensity of the bands was also measured using Image J version 1.29. Oligonucleotide primers for ANP, BNP, αMHC, βMHC, and collagen types I and III are shown in Table I.**

**Quantitative Real-time PCR by SYBR Green Detection Method—The mRNA levels of angiotensin II type 1a receptor (AT1aR), angiotensin II type 2 receptor (AT2R), and transforming growth factor-β1 were evaluated by quantitative real-time PCR.**

**RESULTS**

**Blood Pressure and Heart Rate—Basal levels of systolic blood pressure in WT mice were similar to those of ARKO mice. Systolic blood pressure levels in both WT and ARKO mice were significantly increased by Ang II stimulation, but there was no statistical difference between the two groups (Table II). There was no significant difference in heart rate between WT and ARKO mice, and Ang II stimulation did not affect heart rate in either group (Table II).**

**Reduced Cardiac Mass in ARKO Male Mice with or without Ang II Stimulation—**

**Northern Blot Analysis—**

**RESULTS**

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**Northern Blot Analysis—**

Procedures were performed as previously described (16). In brief, total RNA was isolated from both the right and left ventricles with TRIzol (Invitrogen). RNA concentrations were measured spectrophotometrically at 260 nm, and samples were stored in diethyl dicarbonate-treated water at −80 °C. Approximately 20 μg of total RNA from each sample was fractionated on 1% formaldehyde-agarose gels and transferred to Hybond nylon membranes (Amersham Biosciences) by capillary action in a high salt solution (20 X SSC). Blots were prehybridized in a hybridization solution for 1 h at 42 °C, followed by overnight hybridization with digoxigenin-labeled specific oligonucleotides (DIG Northern Starter Kit, Roche Applied Science). Blots were washed twice in 2 X SSC/0.1% SDS at room temperature for 5 min and then washed twice in 0.2 X SSC/0.1% SDS at 68 °C for 15 min before exposure to X-ray film. Evaluation of mRNA levels encoding A-type natriuretic peptide (ANP), B-type natriuretic peptide (BNP), α-myosin heavy chain (αMHC), β-myosin heavy chain (βMHC), and collagen types I and III was performed. Quantification of mRNA levels was estimated after correction for loading differences by measuring the amount of 28 S rRNA. The intensity of the bands was also measured using Image J version 1.29. Oligonucleotide primers for ANP, BNP, αMHC, βMHC, and collagen types I and III are shown in Table I.
Histomorphometric analyses of the LV tissues showed that the cross-sectional area of cardiomyocytes without Ang II stimulation was significantly smaller in ARKO mice than in WT mice (Fig. 1D). Although Ang II stimulation caused an increase in the cross-sectional area of the cardiomyocytes in both WT and ARKO mice, the cross-sectional area after Ang II stimulation was again smaller in ARKO mice than in WT mice (Fig. 1D).

**Exacerbated Cardiac Fibrosis by Ang II Stimulation in ARKO Male Mice**—The collagen fraction volume ratio calculated from histomorphometric analyses of Masson-Trichrome-stained specimens revealed that cardiac fibrosis was faintly present in LV tissues of WT and ARKO mice without Ang II stimulation. Ang II stimulation elicited a markedly enhanced fibrotic change in LV tissues of ARKO mice compared with those of WT mice (Fig. 1, B, C, and E). However, Ang II administration did not cause an obvious progression of cardiac fibrosis in both WT and ARKO mice when the experiments were conducted at 8 weeks instead of 25 weeks of age (data not shown).

**Analyses of Cardiac Structure**—Echocardiographic studies revealed that Ang II stimulation caused a significant increase in the values of AV and PW in both WT and ARKO mice (mean ± S.E. mm; AV: 0.86 ± 0.03 to 1.15 ± 0.01 in WT and 0.60 ± 0.02 to 0.70 ± 0.02 in ARKO mice; PW: 0.88 ± 0.03 to 1.13 ± 0.03 in WT and 0.68 ± 0.02 to 0.72 ± 0.02 in ARKO mice), whereas there was no statistically significant change in LVM/BW and RWT by Ang II stimulation in ARKO mice. Furthermore, although the values of LVDd and LVDs were decreased in Ang II-stimulated WT mice due to their concentric hypertrophy, these values did not change or even slightly increase in ARKO mice by Ang II stimulation (Fig. 2).

**Ang II-stimulated Cardiac Dysfunction in ARKO Male Mice**—We also evaluated the cardiac performance of these mice using echocardiography. Although left ventricular FS, a marker of systolic function, was not significantly different between WT and ARKO mice, Ang II stimulation significantly attenuated LV systolic function in ARKO mice compared with WT mice (Fig. 2).
pathways have been shown to be involved in cardiac hypertrophy (18, 19) and function (20), we examined effects of Ang II stimulation on ERK1/2 and ERK5 activities. Ang II stimulation caused a significant increase in cardiac ERK1/2 and ERK5 phosphorylation in WT mice, whereas Ang II stimulation did not significantly enhance MAP kinase phosphorylation in ARKO mice (Fig. 3). Phosphorylation of other MAP kinases, c-Jun NH2-terminal kinase and p38, by Ang II were not observed in either WT or ARKO mice (data not shown). These results suggest that the blunted response to Ang II stimulation in ERK1/2 and ERK5 activities may be at least in part related to the aberrant cardiac hypertrophic response and cardiac dysfunction in ARKO mice.

Aberrant Gene Expression of Cardiac Remodeling Factors in ARKO Male Mice—As shown in Fig. 4, the basal gene expression levels of BNP, MHCs, and types I and III collagen were not different between WT and ARKO mice. The ANP mRNA level was slightly lower in ARKO mice than in WT mice. Although Ang II stimulation markedly enhanced the expression levels of ANP and BNP mRNA in both WT and ARKO mice, the expression level of ANP mRNA was increased more in ARKO mice than in WT mice. Ang II stimulation did not affect αMHC mRNA levels in either WT or ARKO mice, but enhanced βMHC mRNA expression only in WT mice. Ang II stimulation enhanced both type I and III collagen mRNA expression levels in WT and ARKO mice, but the enhancement was more pronounced in ARKO mice. Although there were no significant differences in the AT1AR and AT2R mRNA levels between WT and ARKO mice with or without Ang II stimulation, ARKO mice showed a tendency for higher expression in these mRNA levels than WT mice in the presence or absence of Ang II stimulation (Fig. 5).

TGF-β1 Expression and Smad Pathway Were Activated in ARKO Mice by Ang II Stimulation—TGF-β1 has been identified as a contributor of cardiac fibrosis (21), and Ang II is known to affect TGF-β1 expression (22). Upon binding of TGF-β1 to its receptor, phosphorylation of Smad2 and its subsequent translocation to the nucleus is a critical step in TGF-β1 signaling pathway (23, 24). Therefore, we examined the effects of Ang II on TGF-β1 expression and Smad2 activation. Although Ang II stimulation significantly enhanced cardiac TGF-β1 mRNA levels in both WT and ARKO mice, the degree of enhancement of its gene expression was more augmented in ARKO mice than in WT mice (Fig. 5). Consistent with the increase in TGF-β1 mRNA expression, Ang II stimulation also caused a significant increase in cardiac Smad2 phosphorylation in both WT and ARKO mice with more Smad2 phosphorylation in ARKO than in WT mice (Fig. 6).

DISCUSSION

Increased cardiac mass, used synonymously with cardiac hypertrophy, is one of the important cardiovascular risk factors (25). Although appropriate cardiac hypertrophy is an adaptive response to several forms of heart disease, excessive cardiac hypertrophy causes pathological cardiac remodeling. Cardiac mass increases at much greater rates in males than in females from puberty and throughout life, even after allometric adjustment for gender differences in overall body size (26, 27). The gender difference of LV mass has led to the hypothesis that sex hormones, such as androgen and estrogen, influence LV mass (25). In fact, estrogen has a preventive effect on cardiac hypertrophy (28–30), whereas androgen causes cardiac hypertrophy (13, 25, 28–30). In experimental animals, cardiac hypertrophy is suppressed by castration (13, 30, 31) and anti-androgens (32). Serum levels of testosterone, dehydroepiandrosterone, and its sulfate conjugate dehydroepiandrosterone sulfate decrease in males with chronic heart failure (33–36), and administration of testosterone results in an improvement of cardiac function in male patients with congestive heart failure (37). However, it is still unclear whether androgen plays a beneficial role in the regulation of cardiac structure and function.

To clarify the physiological roles of androgen on cardiac structure and function, we used ARKO male mice established using the Cre-loxP system, which allows the AR-null mutation to be passed on to offspring (8, 9). The present study demonstrated that cardiac volume and wall thickness were smaller in ARKO mice than those in WT mice without any difference in blood pressure or heart rate between these mice. The physiological and pathophysiological significance of androgen in male cardiac morphology and function was also examined. Our results indicate that androgen action is required for sufficient cardiac growth with adequate myofiber size, regardless of the presence or absence of cardiac stress in male mice.

Experimental and clinical observations indicate that Ang II
The resulting cardiac hypertrophy is initially a compensatory response to preserve cardiac performance against cardiac load. It is also postulated that the concentric geometric remodeling with a reduction in the LV chamber size relative to wall thickness is an adaptation to preserve the LV pump function (44). The present study demonstrated that Ang II-stimulated ARKO male mice exhibit an aberrant cardiac hypertrophic response to Ang II stimulation. Therefore, our results indicate that AR is required for the physiological hypertrophy of normal postnatal cardiac development in male mice, and for adaptive responses to cardiac stress such as Ang II stimulation.

A critical role for ERK1/2 and ERK5 has been demonstrated in transgenic mice in the development of cardiac hypertrophy (18, 19). In the present study, we found that ERK1/2 and ERK5 were activated by Ang II stimulation in WT male mice, whereas their activities were lower in ARKO male mice after Ang II stimulation. These results are consistent with the macro- and microscopic observations of WT and ARKO male mice hearts, and suggest that ERK1/2 and/or ERK5 activation might be involved in Ang II-induced cardiac hypertrophy. It has been reported that Ang II infusion causes cardiac hypertrophy in rats with a concomitant increase in ERK1/2 activity (45). In addition, ERK5 activation has been shown to cause hypertrophy of cardiomyocytes (46) as well as the inhibition of endothelial cell death (47). Thus, the blunted ERK1/2 and ERK5 activation may be the mechanism whereby ARKO male mice exhibit reduced cardiac size with aberrant hypertrophic response to Ang II stimulation. However, further studies are needed to clarify the precise role of MAP kinases in AR-mediated intracellular signaling in the heart.

The present study demonstrated that the mRNA level of ANP in ARKO mice was reduced compared with WT mice. This result is consistent with the evidence that androgens increase ANP secretion via an AR-mediated mechanism in cultured rat myocytes (11). However, Ang II stimulation caused prominent enhancement of ANP gene expression in ARKO mice more than WT mice. If androgen actions were lacking under cardiac stress, it may be required that some ANP enhancers, including mechanical stress, strongly promote the ANP expression for supporting the cardiac performance. We also found the reduction of βMHC gene expression in Ang II-stimulated ARKO mice compared with WT mice with Ang II loading. An up-regulation of the βMHC gene relative to the αMHC gene is observed in
clinical conditions such as cardiac hypertrophy, cardiomyopathy, and congestive heart failure (48, 49). Because this up-regulation of the gene has been recognized as a compensatory response during cardiac remodeling, we speculate that aberrant βMHC gene expression in ARKO mice is associated with impairment of adaptive cardiac hypertrophy under the condition of hypertrophic stress. Further investigation is needed to clarify this issue. And we found tendencies of higher AT1aR and AT2R gene expression in ARKO mice compared with WT mice, however, there were no statistical differences among those groups. Thus, we could conclude at least in this situation that AT1aR or AT2R expression levels are not a major cause of impaired cardiac hypertrophic response after Ang II stimulation in ARKO mice.

In addition to insufficient cardiac growth and aberrant hypertrophic responses, the present study demonstrated an enhanced cardiac fibrosis with up-regulation of collagen I and III gene expression in ARKO male mice after Ang II infusion. It has been reported that Ang II-induced proliferation of cardiac fibroblasts and an increase of collagen deposition contribute to an increase in cardiac muscle stiffness and the development of diastolic and systolic dysfunctions (50, 51). In fact, the present echocardiographic examination showed that the significant impairment of systolic function was observed in only ARKO male mice with Ang II stimulation. We also found that Ang II stimulation enhanced cardiac TGF-β1 and Smad2 phosphorylation in ARKO mice more than in WT mice. TGF-β1 is a powerful stimulator of the production of fibrillar collagens and other robust expression of TGF-β, which is a major signaling pathway after TGF-β stimulation. This activation of hypertrophic stress. Further investigation is needed to clarify this issue. And we found tendencies of higher AT1aR and AT2R gene expression in ARKO mice compared with WT mice, however, there were no statistical differences among those groups. Thus, we could conclude at least in this situation that AT1aR or AT2R expression levels are not a major cause of impaired cardiac hypertrophic response after Ang II stimulation in ARKO mice.

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