Aromatase Deficiency Confers Paradoxical Postischemic Cardioprotection

James R. Bell, Kimberley M. Mellor, Amanda C. Wollermann, Wendy T. K. Ip, Melissa E. Reichelt, Sarah J. Meachem, Evan R. Simpson, and Lea M. D. Delbridge

Cardiac Phenomics Laboratory (J.R.B., K.M.M., A.C.W., W.T.K.I., M.E.R., L.M.D.D.), Department of Physiology, University of Melbourne, Melbourne 3010, Victoria, Australia; and Prince Henry's Institute of Medical Research (S.J.M., E.R.S.), Monash Medical Centre, Clayton 3168, Victoria, Australia

The conventional view is that estrogen confers female cardioprotection. Estrogen synthesis depends on androgen availability, with aromatase regulating conversion of testosterone to estradiol. Extragonadal aromatase expression mediates estrogen production in some tissues, but a role for local steroid conversion has not yet been demonstrated in the heart. This study's goal was to investigate how aromatase deficiency influences myocardial function and ischemic resilience. RT-PCR analysis of C57Bl/6 mouse hearts confirmed cardiac-specific aromatase expression in adult females. Functional performance of isolated hearts from female aromatase knockout (ArKO) and aromatase wild-type mice were compared. Left ventricular developed pressures were similar in aerobic perfusion, but the maximal rate of rise of ventricular pressure was modestly reduced in ArKO hearts (3725 ± 144 *vs.* 4272 ± 154 mm Hg/sec, $P < 0.05$). After 25 min of ischemia, the recovery of left ventricular developed pressure was substantially improved in ArKO (percentage of basal at 60 min of reperfusion, 62 ± 8 *vs.* $30 \pm 6\%$; $P < 0.05$). Hypercontracture was attenuated (end diastolic pressure, 25 ± 5 *vs.* 51 ± 1 mm Hg; $P < 0.05$), and lactate dehydrogenase content of coronary effluent was reduced throughout reperfusion in ArKO hearts. This was associated with a hyperphosphorylation of phospholamban and a reduction in phosphorylated Akt. Immediately after reperfusion, ArKO hearts exhibited increased incidence of ventricular premature beats (194 ± 70 *vs.* 46 ± 6, $P < 0.05$). These observations indicate more robust functional recovery, reduced cellular injury, and modified cardiomyocyte Ca^{2+} handling in aromatase-deficient hearts. Our findings indicate that androgen-to-estrogen conversion may be of pathophysiologic importance to the heart and challenge the notion that estrogen deficiency is deleterious. These studies suggest the possibility that aromatase suppression may offer inotropic benefit in the acute ischemia/reperfusion setting with appropriate arrhythmia management. **(*Endocrinology* 152: 4937–4947, 2011)**

Sex differences in cardiovascular disease have been attributed to the modulatory actions of gonadally derived sex hormones. The conventional view is that estrogen affords a level of cardiovascular protection in women, whereas an androgenic influence is generally considered a cardiovascular liability in men. However, there is accumulating evidence to indicate that the actions of these sex hormones on the heart may be more complex than would be suggested by this simple contrast.

Animal models of ischemia/reperfusion have shown improved myocardial viability and functional recovery in female hearts compared with males (1–3), although experimental design factors, including the length of ischemia, may be crucial in determining the occurrence of immediate postischemic protection (4). Systemic estrogen suppression through ovariectomy generally diminishes functional recovery and increases injury in ischemia/rep-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2011-1212 Received May 7, 2011. Accepted September 15, 2011.
First Published Online October 25, 2011

Abbreviations: ArKO, Aromatase-knockout; ArWT, aromatase wild type; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2; bpm, beats per minute; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; dP/dt max, maximal rate of rise of ventricular pressure; dP/dt min, minimal rate of decline in ventricular pressure; LDH, lactate dehydrogenase; LV DevP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; ns, not significant; PI3-K, phospho-inositide-3 kinase; PKA, protein kinase A; PLB, phospholamban; SERCA2a, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a; SR, sarcoplasmic reticulum; VPB, ventricular premature beat; VWI, ventricular weight index.

erfusion. This effect may be reversed with chronic estrogen administration (5–9), but in some contexts, estrogen supplementation has been found to exacerbate injury (10, 11). Other studies provide evidence of a protective role for testosterone in ischemia/reperfusion. Chronic testosterone supplementation in male rat hearts increases postischemic function (12) and improves survival (13). Furthermore, dihydrotestosterone improves functional recovery in ovariectomized female rats to levels similar to that observed in control, intact females (14). The role of estrogens and androgens in cardiac ischemia has yet to be clearly defined, and a more nuanced understanding is required.

Estrogen production depends on androgen availability, and the levels of these two steroids are linked through the enzymatic actions of aromatase (*CYP19a1*), converting testosterone to estradiol. Although ovaries are the primary source of systemic estrogen in females, numerous cell types have also been shown to express aromatase and produce estrogen (*e.g.* bone and adipocytes), exerting a paracrine/autocrine action in the milieu of fluctuating circulating hormone levels (15). Aromatase has been shown to be expressed in immature hearts/cardiomyocytes (16, 17), suggesting potential for the presence of intracardiac androgen-estrogen conversion. Aromatase expression in the adult myocardium has not been previously identified.

Previous studies assessing the effects of chronic sex hormone manipulation on the ischemic heart have focused on systemic, gonadally derived sex hormones. No studies to date have considered the possibility that suppression of androgen conversion to estrogen in nongonadal tissues, including the heart, may impact on myocardial function. Using a female genetic model of aromatase deficiency, the goal of this study was to investigate the effects of global estrogen withdrawal (in all tissues, including cardiac and gonadal) on the *ex vivo* heart responses to ischemia/reperfusion challenge. Surprisingly we have found that in the estrogen-deficient/testosterone replete state, cardiac mechanical function is markedly enhanced post-ischemia/reperfusion (although in a context of increased arrhythmogenesis). Our studies indicate that androgen-to-estrogen conversion may be of pathophysiologic importance and suggest the possibility that estrogen suppression may offer inotropic benefit in the acute ischemia/reperfusion setting with appropriate arrhythmia management.

Materials and Methods

Animals

Aromatase-deficient [aromatase knockout (ArKO)] mice were generated by targeted disruption of the *CYP19* gene, as described (18). Age-matched female C57Bl/6, ArKO, and aromatase wild-type (ArWT) mice (20 wk) were maintained at the Biomedical Sciences Animal Facility at the University of Melbourne. All animals were handled in the manner specified by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004), and this project was approved by the University of Melbourne Animal Ethics Committee (no. 0811022). Mice were fed a phytoestrogen-free diet (AIN93G; Specialty Feeds, Glen Forrest, Australia) from approximately 6 wk of age.

Isolated heart preparation

Mice were anesthetized with sodium pentobarbitone in combination with sodium heparin (200 mg/kg and 200 IU/kg, respectively, ip). Hearts were rapidly excised, placed in cold (4 C) Krebs-Henseleit bicarbonate buffer, and the aorta cannulated. The hearts were retrogradely perfused with oxygenated (95% O_2, 5% CO_2) bicarbonate buffer (37.0 C, pH 7.4) in the nonrecirculating Langendorff mode at a constant pressure equivalent to 80 mm Hg using a pump controller (STH model; ADInstruments, Bella Vista, Australia) (19). The bicarbonate buffer contained 119 mmol/liter NaCl, 4.0 mmol/liter KCl, 1.2 mmol/liter KH_2PO_4, 22.0 mmol/liter $NaHCO_3$, 1.2 mmol/liter $MgCl_2$, 2.5 mmol/liter $CaCl_2$, 0.5 mmol/liter EDTA, 2.0 mmol/liter Na pyruvate, and 5.0 mmol/liter glucose. Coronary flow was measured continuously throughout the perfusion protocol. An initial subgroup of hearts was not electrically paced and was allowed to beat spontaneously.

In a subsequent set of ischemia/reperfusion experiments, hearts were aerobically perfused for 30 min before 25 min of global ischemia (37.0 C) and 60 min of reperfusion. Hearts were paced at 580 beats per minute (bpm) throughout the stabilization period and for the first 2 min of ischemia. Pacing commenced at 5 min of reperfusion. Left ventricular pressure measurements were performed using a fluid-filled balloon connected to a pressure transducer (MLT844) and recorded on a MacLab data acquisition system (ADInstruments). The balloon was inflated to produce an end-diastolic pressure of 5–9 mm Hg and the volume kept constant throughout the perfusion protocol. Parameters measured included left ventricular developed pressure (LV DevP), heart rate, left ventricular end diastolic pressure (LVEDP), and the maximal rate of rise and decline of ventricular pressure (dP/dt max and dP/dt min, respectively). Left ventricular pressure traces were analyzed for the incidence of ventricular arrhythmias, as detailed (20). Arrhythmic activity was scored in a blinded manner for the first 5 min of reperfusion. Coronary effluent was collected throughout reperfusion for subsequent lactate dehydrogenase (LDH) analysis. At the end of reperfusion, ventricles were snap frozen in liquid nitrogen for molecular analyses.

LDH assay

LDH analysis of coronary effluents collected throughout reperfusion was performed using a spectrophotometric LDH assay [Cytotoxicity Detection kit-PLUS (LDH); Roche, Basel, Switzerland], as described elsewhere (21). Briefly, coronary effluent samples were thawed and assayed against LDH standards (L-lactic dehydrogenase solution, L2625; Sigma-Aldrich, St. Louis, MO). Total LDH released from the heart throughout reperfusion was expressed as effluent concentration (IU) per gram of wet ventricular weight.

Cardiac aromatase gene expression analysis

Ventricular and ovarian RNA was extracted from (nongenetically modified) control female C57Bl/6 mouse tissues and reverse transcribed as described (22). Relative gene expression levels of cardiac aromatase (*i.e. CYP19A1*; accession no. NM_007810) and the housekeeper gene (18S) in ventricular and ovarian tissue were evaluated by real-time RT-PCR as previously described (22, 23). Conventional RT-PCR was performed with two consecutive runs of 30 PCR cycles to allow visualization of low expression genes. Primer sequences were: aromatase (*CYP19A1*; real-time PCR), 5′-TGGACGAAAGTGCTATTGTGAA-3′ and 5′-TCTTTCAAGTCCTTGACGGAT-3′; 18S (real-time PCR), 5′-TCGAGGCCCTGTAATTGGAA-3′ and 5′-CCCTCCAATGGATCCTCGTT-3′; and aromatase (*CYP19A1*; conventional PCR), 5′-ATCCGTCAAGGACTTGAAAGA-3′ and 5′-TGTAGGGAACATTCTTCTCAA-3′.

Protein analysis and Western blotting

All tissues (cardiac, ovary, placenta, and adipose) were thawed and homogenized (10% wt/vol) in a Tris-HCl 100 mM, EGTA 5 mM, EDTA 5 mM (Sigma-Aldrich) buffer containing protease and phosphatase inhibitors (Roche) at 4 C and added to 2× SDS sample buffer. Ventricular sample protein concentrations were determined with a Bradford assay (595 nm) (Bio-Rad Laboratories, Gladesville, Australia). Equal amounts of protein were then loaded onto 10–15% polyacrylamide gels and SDS-PAGE and subsequent Western blot analysis performed, as detailed (2). Loading of non-cardiac homogenates (10% wt/vol) was by equal volume and visualized by Ponceau staining (Supplemental Fig. 1, published on the Endocrine Society's Journals Online web site at http://endo.endojournals.org). Equal loading of cardiac homogenate samples was verified by subsequent densitometric scanning (Bio-Rad Chemi-XRS Imaging, Bio-Rad Laboratories) of Ponceau-stained (Ponceau-S, Sigma, St. Louis, MO) membranes (Supplemental Fig. 2).

Primary antibodies used in this study included aromatase (Ar677; Novartis Pharma AG, Basel, Switzerland), sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) (A010-20; Badrilla, Leeds, UK), total phospholamban (PLB) (05-205; Upstate, Waltham, MA), phospho(Ser16)-specific PLB (07-052; Upstate), phospho(Thr17)-specific PLB (A010-13; Badrilla), total Akt (9272; Cell Signaling, Beverly, MA), phospho-(Ser473)-specific Akt (9271; Cell Signaling), Bcl-2 associated X protein (Bax) (2772; Cell Signaling), and B-cell lymphoma 2 (Bcl-2) (2876; Cell Signaling).

Cardiomyocyte isolation and dimension measurements

Ventricular cardiomyocytes were isolated from ArWT and ArKO mice (n = 4–7), using enzymatic digestion with Type II Collagenase (0.66 mg/ml, 295 U/mg; Worthington Biochemical Corp., Lakewood, NJ) and trypsin (33 μg/ml; Sigma-Aldrich) (24). Cardiomyocytes were resuspended in normal potassium, Ca^{2+}-free HEPES-buffered Krebs and maximal cell width and length measured under ×400 magnification (Nikon, Tokyo, Japan) using a calibrated graticule eyepiece (n = 50 cells/heart).

Statistics

Data are presented as mean ± SEM. Differences between groups were assessed using a Student's unpaired *t* test or a one-way ANOVA with repeated measures as appropriate. Data were considered significant at $P < 0.05$. All statistical calculations were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL).

Results

Cardiac-specific aromatase expression

Evidence of cardiac aromatase expression in normal adult myocardium was sought. Real-time RT-PCR analysis of mRNA extracted from C57Bl/6 female (nongenetically modified) hearts demonstrated the presence of a *CYP19A1* gene transcript (Fig. 1A). Transcript examination by conventional PCR confirmed that the amplified transcript corresponded with the predicted gene product of 535 bp (Fig. 1B). Further studies confirmed aromatase mRNA translation, with Western blot analysis showing that aromatase protein was present in the adult heart (Fig. 1C). Cardiac aromatase was expressed at relatively low level compared with placenta and adipose; it is important to note that those nongonadal tissues are recognized to have particularly prominent local aromatase activity.

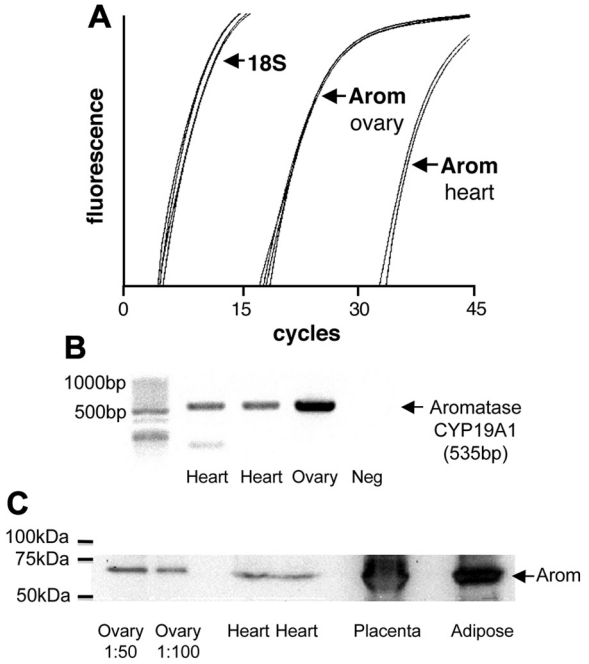

FIG. 1. Cardiac aromatase expression. In panel A, the real-time PCR fluorescence depicts aromatase (*Arom*) gene expression (*Cyp19a1*) relative to 18S in female mouse heart and ovarian (positive control) tissue. Panel B depicts the DNA gel image from conventional RT-PCR of aromatase (*Cyp19a1*) in female mouse heart tissue. Aromatase primers were designed to obtain a 535 bp PCR product. Ovarian tissue was used as a positive control. Negative control (neg) was obtained by RNA that was not reverse transcribed to cDNA. Panel C shows aromatase protein expression in cardiac tissue. Expression levels are relatively low compared with placenta and adipose tissue (nongonadal tissues with particularly prominent local androgen-to-estrogen systems). Ovarian tissue (diluted) was used as a positive control.

Evaluation of these expression differences cannot be highly quantitative, because the protein composition and proportion in different tissues vary considerably according to structure/cellular heterogeneity. The "loading control" in these disparate tissues is essentially a control relative to tissue mass (see Supplemental Fig. 1), and the Western blot analysis provides an indicative comparison of nongonadal tissue aromatase expression. The identification of myocardial aromatase expression in adult females suggests potential for global aromatase suppression/deletion to impact on local cardiac estrogen generation.

Normal heart growth in aromatase-deficient hearts

The effect of aromatase deficiency on cardiac growth was evaluated by measurement of cardiac weight and isolated ventricular cardiomyocyte dimensions (Table 1). ArKO ventricular weight index (VWI) was significantly decreased compared with ArWT controls (6.1 ± 0.2 *vs.* 7.1 ± 0.3 mg/g, $P < 0.05$), although no change in heart weight was evident. The reduced VWI reflects changes in body mass (23.9 ± 1.0 *vs.* 21.0 ± 0.9 g, $P = 0.059$), because ArKO mice exhibit increased intraabdominal adipose tissue, as previously described (25). Analysis of the dimensions of enzymatically isolated viable cardiomyocytes confirmed this, with no differences observed between genotypes in cell length, width, or area (Table 1), attesting to a lack of cardiotrophic modulation in the ArKO model.

Cardiac function in aerobic and ischemic stress conditions

To assess the effect of global aromatase deficiency on basal myocardial performance, the contractile function of isolated hearts was assessed during aerobic perfusion. No difference between ArKO and ArWT in intrinsic heart rate was observed in spontaneously beating (nonpaced) hearts [465 ± 10 *vs.* 467 ± 13 bpm, P = not significant (ns)].

TABLE 1. Dimensions of cardiomyocytes from ArKO and ArWT mice

	ArWT	ArKO
Body weight (g)	21.0 ± 0.9	23.9 ± 1.0
Ventricular weight (mg)	149 ± 2	146 ± 4
VWI (mg/g)	7.1 ± 0.3	6.1 ± 0.2[a]
Cardiomyocyte width (μm)	26.9 ± 0.4	27.5 ± 0.3
Cardiomyocyte length (μm)	127 ± 1	129 ± 1
Cardiomyocyte area (μm^2)	3443 ± 72	3592 ± 58

Data presented as mean ± SEM (body/ventricular weights, $n = 5$; cardiomyocyte measurements, n = ~50 cells/mouse; $n = 4$–7 mice/group). Ventricular weights are wet weights at the end of reperfusion.
[a] $P < 0.05$, ArKO *vs.* ArWT.

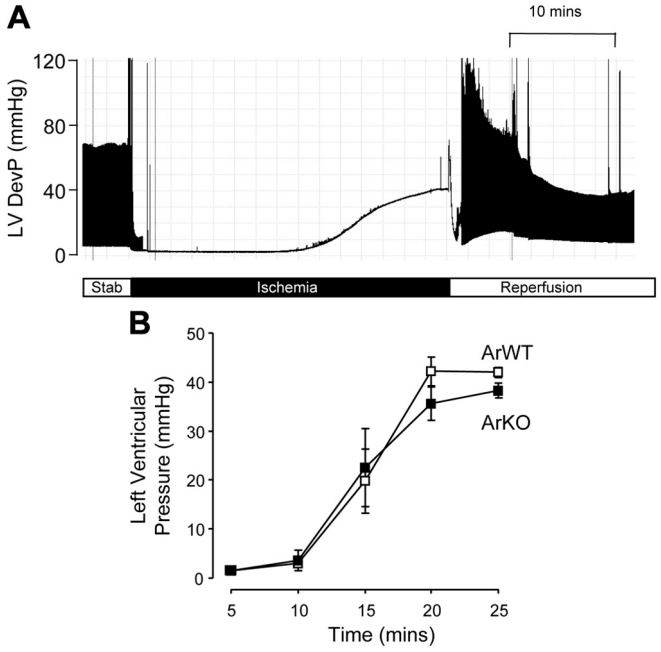

FIG. 2. Left ventricular pressure traces in ischemia/reperfusion. A representative pressure trace is shown in A, incorporating the end of the stabilization period (Stab), ischemia, and the first 15 min of reperfusion. No difference in the onset and amplitude of ischemic contracture was observed between genotypes (B). Data are mean ± SEM (n = 5), analyzed by a one-way ANOVA with repeated measures, P = ns.

Figure 2A shows a representative left ventricular pressure trace for a cohort of electrically paced ArWT and ArKO hearts during aerobic perfusion, ischemia, and subsequent reperfusion. No genotype differences were observed in LV DevP, dP/dt min, and coronary flow during the preischemic stabilization period (Table 2), but a small, significant decrease in dP/dt max was evident in ArKO hearts (3725 ± 144 *vs.* 4272 ± 154 mm Hg/sec, $P < 0.05$). These results suggest that aromatase deficiency had minimal effects on basal cardiac function. The responses of ArWT and ArKO hearts to ischemic insult were compared, assessing the onset and amplitude of ischemic contracture as an index of injury. Figure 2 shows that ArWT and ArKO hearts exhibited similar contracture profiles throughout ischemia.

TABLE 2. Basal cardiac function in ex vivo ArKO and ArWT heart

	ArWT	ArKO
LV DevP (mm Hg)	77 ± 3	70 ± 2
dP/dt max (mm Hg/s)	4272 ± 154	3725 ± 144[a]
dP/dt min (mm Hg/s)	−2666 ± 120	−2441 ± 112
Coronary flow (mg/ml)	2.7 ± 0.3	2.6 ± 0.6

Data presented as mean ± SEM ($n = 5$ mice/group). Values are functional parameters at 25-min reperfusion.
[a] $P < 0.05$, ArKO *vs.* ArWT.

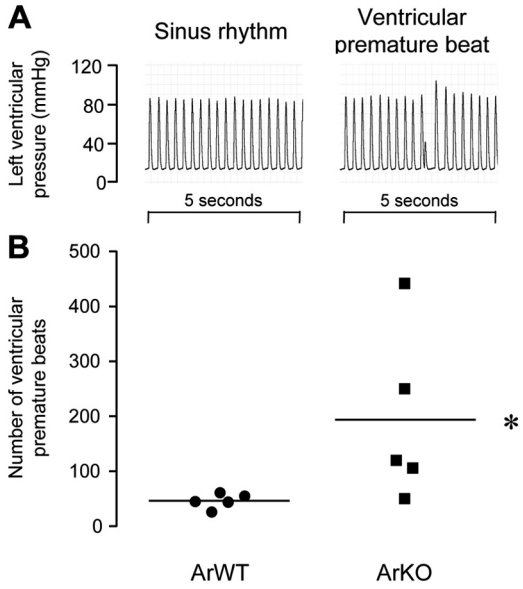

FIG. 3. Incidence of VPB in early reperfusion. A, Examples of left ventricular pressure traces demonstrate a heart in sinus rhythm and during a VPB. B, The number of VPB in the first 5 min of reperfusion was significantly increased in ArKO compared with ArWT controls. Data are mean ± SEM (n = 5), analyzed by Student's *t* test; *, $P < 0.05$.

Incidence of reperfusion-induced arrhythmias

Detailed examination of the early reperfusion phase was undertaken because this period is typically characterized by cardiomyocyte Ca^{2+} overload, contractile dysfunction, and occurrence of ventricular arrhythmias. The incidence and type of arrhythmias were assessed in the first 5 min of reperfusion. Hearts were not electrically stimulated during this period, and the total number of beats was similar for ArKO and ArWT genotypes (1267 ± 43 *vs.* 1260 ± 92 bpm, P = ns). Detailed analysis of arrhythmia types revealed that ArKO hearts exhibited a higher propensity for ventricular premature beats (VPB) compared with ArWT controls (193.6 ± 70.2 *vs.* 46.2 ± 6.0, $P < 0.05$), indicative of augmented SR Ca^{2+} loading (Fig. 3).

Improved postischemic functional recovery in reperfusion

Contractile function was tracked throughout reperfusion to assess the effect of aromatase deficiency on cardiac mechanical recovery from an ischemic insult. Throughout reperfusion, marked differences in systolic and diastolic function were apparent between ArKO and ArWT hearts (Fig. 4). Enhanced systolic performance (LV DevP, dP/dt max) (Fig. 4A) was associated with a concomitant improvement in diastolic function (dP/dt min, LVEDP) (Fig. 4B). Percentage recovery (relative to preischemic basal) (Fig. 5) of systolic function was markedly higher, with LV DevP (62 ± 8 *vs.* 30 ± 6%, $P < 0.05$) and dP/dt max (79 ± 12 *vs.* 30 ± 5%, $P < 0.05$) in ArKO hearts more than double the levels observed in ArWT controls (Fig. 5, A and B). dP/dt min was increased (63 ± 9 *vs.* 30 ± 4%, $P < 0.05$) (Fig 5C) and LVEDP elevation in reperfusion was less marked in ArKO hearts compared with ArWT (25 ± 5 *vs.* 51 ± 1 mm Hg, $P < 0.05$) (Fig 4B), suggesting an improved capacity to manage cytosolic Ca^{2+} removal in relaxation. Overall, both systolic and diastolic function were significantly improved in ArKO hearts.

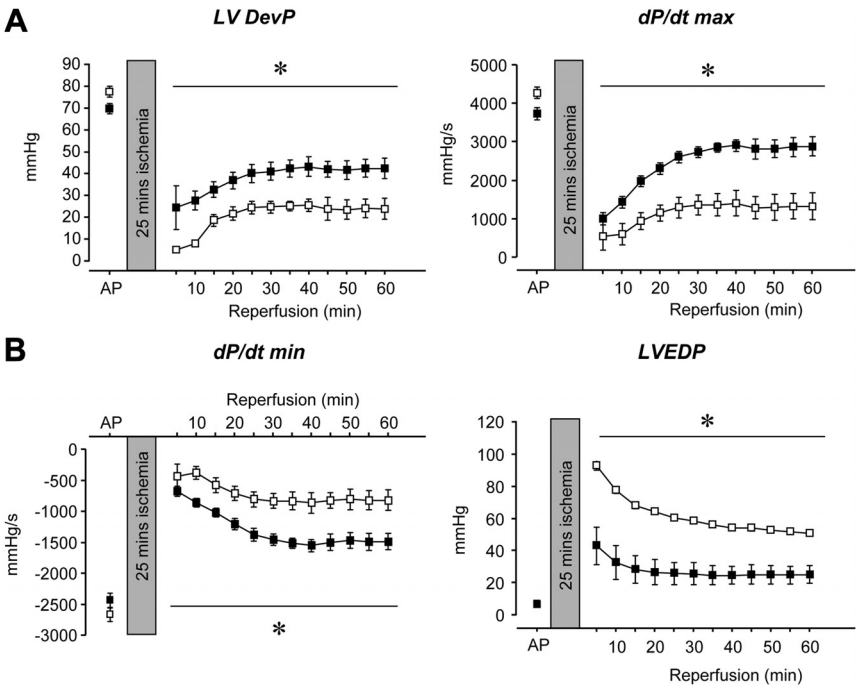

FIG. 4. Time course of contractile function throughout 60 min of reperfusion. Systolic performance (LV DevP and dP/dt max) (A) was increased throughout the ischemia/reperfusion protocol in ArKO hearts (ArWT, □; ArKO, ■). Similarly, diastolic function was improved (B), with dP/dt min augmented and the extent of increase in LVEDP attenuated. Data are mean ± SEM (n = 5), analyzed by a one-way ANOVA with repeated measures; *, $P < 0.05$.

Differential myocyte viability in reperfusion

During reperfusion, cardiomyocyte hypercontracture, cell swelling and calpain activation, compromises cell integrity and is associated with the release of intracellular LDH. The extent of LDH release linearly correlates with infarct size (26) and is therefore used as a marker of nonprogrammed cardiomyocyte death. A substantial reduction in LDH release in ArKO hearts compared with ArWT controls (1.0 ± 3.4 *vs.* 22.4 ± 7.9 IU/g, $P < 0.05$) was observed (Fig. 6A).

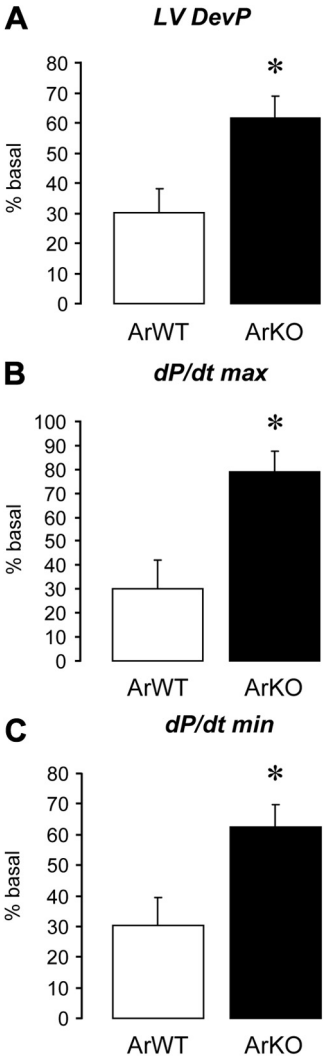

FIG. 5. Percentage recovery of contractile function at the end of 60 min of reperfusion. Recovery at the end of reperfusion was assessed as a percentage of the preischemic basal value. Recovery of LV DevP, dP/dt max, and dP/dt min (A–C) were more than doubled in ArKO hearts compared with ArWT controls. Data are mean ± SEM (n = 5/group), analyzed by Student's *t* test; *, $P < 0.05$.

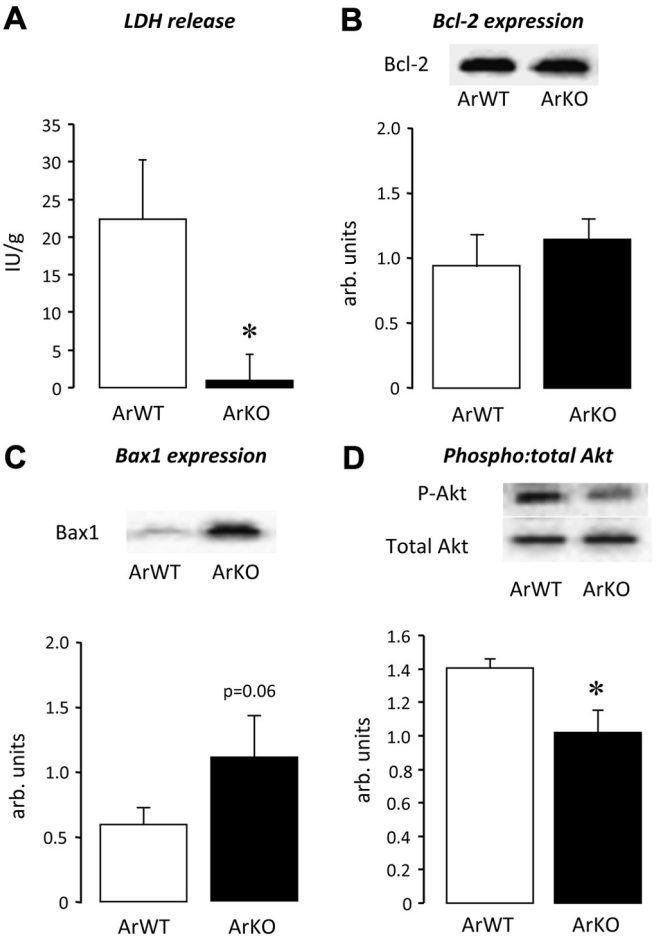

FIG. 6. Cell death in reperfusion. Coronary effluent was collected throughout 60 min of reperfusion and LDH content measured as a marker of cell lysis. A, Significant reduction in LDH release in ArKO hearts. Hearts frozen at the end of reperfusion were analyzed for markers of apoptosis and cell survival signaling. No genotypic difference was observed in the protein expression of the antiapoptotic marker, Bcl-2 (B). An increase in proapoptotic Bax expression in ArKO hearts failed to reach significance ($P = 0.06$) (C) but was associated with a significant reduction in the phospho(Ser473):total ratio of Akt (D). Data are mean ± SEM (n = 5/group), analyzed by Student's *t* test; *, $P < 0.05$. arb. units, Arbitrary units.

Myocardial gene expression of the apoptosis (programmed cell death) regulators, Bax (proapoptotic) and Bcl-2 (antiapoptotic), was evaluated. No change was observed in the antiapoptotic marker Bcl-2 (Fig. 6C) or the Bax:Bcl-2 ratio (data not shown), although a nonsignificant increase in Bax expression in ArKO hearts (arbitrary units, 1.11 ± 0.32 *vs.* 0.60 ± 0.15; $P = 0.06$) (Fig. 6C) suggests a proapoptotic trend at 60 min of reperfusion (loading controls shown in Supplemental Fig. 2). Further studies using chronic reperfusion protocols (>1 h) are indicated to fully characterize the effects of aromatase deficiency on postischemic apoptosis activation.

Cardioprotection associated with estrogen is proposed to be mediated through phospho-inositide-3 kinase (PI3-K)-activated Akt, stimulating nitric oxide production and minimizing mitochondrial permeability transition pore opening (27). The phosphorylation status of Akt was therefore determined in reperfused ArKO hearts to assess the effects of a change in the balance of estrogen and testosterone (Fig. 6D). Total Akt expression was unchanged. However, Akt phosphorylation was significantly decreased in ArKO hearts (phospho:total ratio, 1.02 ± 0.14 *vs.* 1.40 ± 0.06; $P < 0.05$), as would be expected in a model of estrogen suppression (28).

Modulated intracellular Ca^{2+} store uptake function in reperfusion

Sarcoplasmic reticulum (SR) Ca^{2+} sequestration via SERCA2a pump activity is directly regulated by the accessory protein, PLB. Phosphorylation of PLB at either the

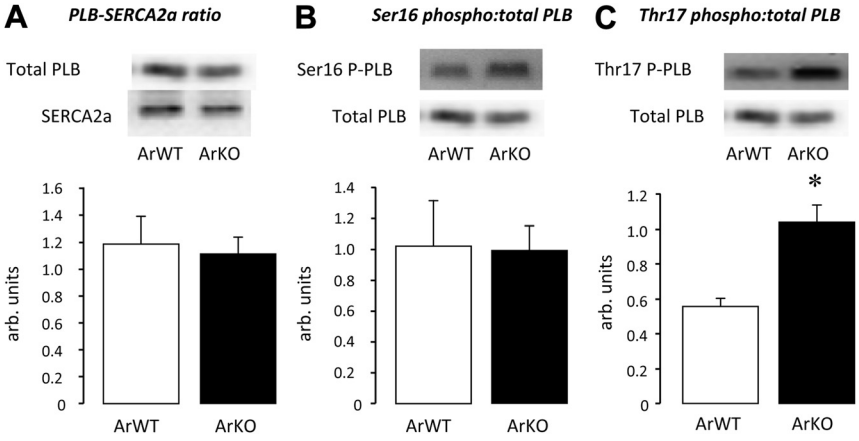

FIG. 7. Modified intracellular Ca^{2+} store uptake function in reperfusion. Hearts frozen at the end of reperfusion were subjected to SDS-PAGE/Western blotting. No change was observed in the expression ratio of PLB:SERCA2a (A), nor in the phosphorylation status of PLB at the PKA-specific Ser16 residue (relative to total PLB expression) (B). However, C shows that the phospho(Thr17):total ratio of PLB (CaMKII-specific site) was significantly increased in ArKO hearts. Data are mean ± SEM (n = 5/group), analyzed by Student's *t* test; *, $P < 0.05$. arb. units, Arbitrary units.

Ser16 or Thr17 residues relieves the inhibitory actions of PLB on SERCA2a, thereby stimulating Ca^{2+} uptake into the SR. Under normoxic conditions, no differences in total SERCA2a or PLB expression, nor the phosphorylation status of PLB, were observed between ArWT and ArKO (data not shown). At the end of the reperfusion protocol, the expression ratio of PLB to SERCA2a remained unchanged (Fig. 7A). Interestingly, ArKO hearts exhibited no change in phosphorylation status at the protein kinase A (PKA)-specific Ser16 site on PLB (Fig. 7B) but an increased phosphorylation of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-specific Thr17 site (arbitrary units, relative to total PLB expression: 1.04 ± 0.10 *vs.* 0.56 ± 0.04; $P < 0.05$) (Fig. 7C). This CaMKII-mediated increase in SR Ca^{2+} loading via SERCA2a in reperfusion may be responsible for the increased postischemic contractile function in ArKO hearts.

Discussion

This investigation is the first to show that aromatase expression can be detected in normal adult female myocardial tissues and that global expression suppression profoundly alters the cardiac response to ischemia and reperfusion, enhancing myocardial function and cardiomyocyte viability in the immediate postischemic period. Although basal function in female *ex vivo* hearts was only subtly modulated by aromatase deficiency in the ArKO model, systolic and diastolic functional recovery in reperfusion was augmented, and the extent of contracture-related cardiomyocyte lysis was attenuated. However, this more robust inotropic recovery in the ArKO was linked with a greater incidence of reperfusion-induced arrhythmias and associated with molecular evidence of altered cardiomyocyte Ca^{2+} handling. These findings suggest that inotropic functional benefit may be conferred on the ischemic/reperfused heart by an alteration in the capacity for androgen-to-estrogen conversion *in vivo*, albeit coupled with increased arrhythmogenic potential.

Functional implications of aromatase deficiency

We have previously described the distinct actions of sex steroids on isolated cardiomyocyte electromechanical function, reporting testosterone augmentation and estrogen attenuation of contractility and cytosolic Ca^{2+} fluxes (29, 30). Based on the systemic hormone levels in the ArKO (negligible estradiol and elevated testosterone) (18), we anticipated elevated contractile performance in aerobically perfused hearts compared with ArWT. Surprisingly, only very subtle differences were observed in systolic parameters in paced hearts, and we infer that the influence of sex steroids on myocyte function in the intact heart is moderated. Further studies involving isolated ArKO cardiomyocytes could provide insight into the inherent alterations of excitation-contraction coupling occurring in these hearts. Intrinsic heart rate was not changed in *ex vivo* spontaneously beating hearts, contrasting with the positive chronotropy reported in ArKO hearts *in vivo* (31). These findings indicate that the central control of heart rate is influenced by altered androgen-estrogen balance but not local pacemaker-driven activity.

Cardioprotective actions for both estrogen and testosterone?

The ArKO mouse has suppressed plasma estrogen and elevated testosterone levels (18). The significantly more robust contractile performance observed in the immediate postischemic period in the ArKO mouse heart is therefore inconsistent with the conventional cardiovascular view that estrogen is beneficial and testosterone detrimental. Age-related changes in the susceptibility of women to cardiovascular disease have been attributed to a reduction in systemic estrogen levels after menopause (32). However, hormone replacement trials have not demonstrated a benefit for postmenopausal women in primary or secondary prevention, and the Women's Health Initiative Study was terminated early due to a small, but significant, increase in

ischemic cardiovascular events (33, 34). Similar anomalies are also evident in the experimental literature relating to various animal models. Isolated hearts from ovariectomized rodents exhibit decreased postischemic contractile performance and enhanced injury (3, 5, 6, 8), an effect reversed with estrogen administration (5–8). Similarly, poor postischemic outcomes are observed with genetic deletion of cardiac estrogen receptor expression (35–37). However, numerous studies report no female/estrogen cardioprotection (38–41), and in some instances, estrogen is implicated in long-term compromised outcomes (10, 11). Estrogen capacity to confer benefit may be dependent on several factors, including the severity of the ischemic insult, animal age, and cardiomyocyte Ca^{2+} handling status (37, 42–44).

There is a developing evidence base to suggest that the capacity for positive testosterone influence may be underrecognized and indeed that testosterone may have a beneficial role in ischemia/reperfusion in both males (12, 13) and females (14). In females, a limitation of the ovariectomy model is that it also eliminates a primary systemic source of testosterone; thus, poor outcomes associated with the removal of ovaries may be attributable to a withdrawal of both/either estrogen or testosterone. In ovariectomized rodents, it has been shown that comparable restoration of postischemic function can be achieved with estrogen or with 5α-dihyrdotestosterone (nonaromatisable androgen) supplementation (14). This study now extends that earlier finding to show that in a genetic model of estrogen suppression and testosterone elevation (systemic and tissue), female hearts exhibit enhanced contractile recovery and maintain cardiomyocyte integrity in the acute postischemic period. It hence seems feasible that there is potential for both estrogen and testosterone beneficial action in a range of settings, using distinct signaling intermediates to target different functional and cell survival endpoints.

Improved ArKO recovery in early reperfusion—mechanistic insights

We have previously shown that testosterone augments isolated cardiomyocyte Ca^{2+} handling and contractility under basal conditions (30). Consistent with this, androgens have been reported to provide inotropic support in specific conditions of contractile dysfunction (45, 46). Testosterone increases the inotropic response and facilitates SR Ca^{2+} release and uptake activity in adrenergically stimulated hearts, promoting PLB phosphorylation (at the PLB Thr17 site) (47). In the present study, the improved contractile performance in postischemic ArKO hearts was similarly associated with a hyperphosphorylation of the CaMKII-specific PLB Thr17 residue, although no genotypic change was observed in PLB Ser16 phosphorylation. This suggests that CaMKII is up-regulated in reperfused ArKO hearts independently of a PKA-mediated increase in cytosolic Ca^{2+} and that CaMKII activity may be influenced by sex steroidal levels in ischemic stress conditions.

PLB phosphorylation at either residue is well characterized to exert a stimulatory effect on SR Ca^{2+} uptake mediated by the SERCA2a pump. Although up-regulated SERCA2a activity augments diastolic SR Ca^{2+} uptake and increases the Ca^{2+} available for contraction, it can also be proarrhythmogenic. SR Ca^{2+} overloading is linked to spontaneous Ca^{2+} release episodes and elevated frequency of postischemic arrhythmias *in vivo* (48, 49). Hence, the higher levels of testosterone in ArKO hearts may increase SR loading through increased PLB phosphorylation to enhance contraction in reperfusion, but potentially at the expense of escalating spontaneous, arrhythmogenic events.

The reduction in LDH release in ArKO hearts was surprising, especially in the context of decreased postischemic Akt phosphorylation. LDH release in reperfusion occurs secondary to hypercontracture and sarcolemmal rupture and closely correlates with infarct size (26). Removal of testosterone has been shown to increase LVEDP in reperfused male rat hearts, particularly in early reperfusion (12), consistent with the improved diastolic performance that we observed in ArKO hearts. This improved diastolic function and diminished hypercontracture indicate that a testosterone-mediated up-regulation in SR Ca^{2+} uptake may be responsible for the reduced LDH release in ArKO hearts. This is interesting, given the finding that Akt phosphorylation was diminished in reperfusion, because this signaling intermediate is implicated in protection against hypercontracture and postischemic tissue salvage through the PI3-K signaling pathway (50, 51). Increased Akt phosphorylation in female myocardium (52) is understood to be a mechanism by which estrogen may exert protection against ischemia/reperfusion injury (53, 54), stimulating nitric oxide synthesis and limiting mitochondrial permeability pore opening (27). Our new finding suggests that both sex steroids may mediate cardioprotection but through distinct mechanisms: testosterone enhancing Ca^{2+} management and estrogen up-regulating PI3-K/Akt. This may be a context-sensitive balance. Earlier studies by Murphy and co-workers (37, 43, 44) showed that poor outcomes in postischemic males (relative to females) were only observed when cardiomyocytes were Ca^{2+} loaded. Beneficial actions of testosterone may therefore be lost when this up-regulated capacity to handle Ca^{2+} is overwhelmed. Hence, the severity of the ischemic insult may determine which sex steroid provides the optimal mechanism for managing the cellular response and indicates

that the balance of androgens and estrogens may critically influence the acute and long-term outcomes.

A functional role for a local myocardial androgen-estrogen system?

Multiple extragonadal cell types have been shown to express aromatase and to facilitate tissue-specific local estrogen production (*e.g.* bone and adipocytes) (15). Dependent on circulating precursor C_{19} steroids, these local androgen-estrogen "systems" are thought to exert significant intracrine/paracrine action and may be influential in tissue-specific disease settings. Analagous to the well-established intracardiac renin-angiotensin system, which mediates important local hormone actions in a milieu of systemic fluctuation, a local androgen-estrogen system could be postulated to operate in the heart. Aromatase expression in adult murine myocardium (Fig. 1) corroborates previously reported findings of cardiac-specific aromatase expression in immature hearts/cardiomyocytes (16, 17) and is consistent with the concept of an intracardiac androgen-estrogen system. Identification of the cellular source(s) of adult myocardial aromatase expression remains elusive, although studies in cultured neonatal cardiomyocytes (17) suggest this cell type as a probable candidate. Alternatively, cardiac fibroblasts (highly populous in the heart) or coronary vasculature may express aromatase to exert local actions in the heart. Indeed, analysis of female adipose tissue revealed that aromatase expression was preferentially expressed in adipose stromal cells compared with adipocytes (55). Further studies will determine whether a similar aromatase expression profile exists between cardiomyocytes and cardiac fibroblasts.

We are not aware of any studies that have directly manipulated local cardiac androgen/estrogen levels; hence, the ArKO mouse provides a unique model to interrogate this proposition. Although the global disruption of aromatase activity in the ArKO mouse does not allow a specific delineation of local and systemic actions, the findings do suggest a basis for a more detailed exploration of local hormone action.

The improved functional recovery and cellular integrity in postischemic ArKO hearts contrast with that observed in hearts of ovariectomized animals. This may be related to fundamental differences in the systemic and/or local sex steroidal levels of ArKO and ovariectomized adult mice. However, as with all genetic deletion models, we cannot exclude the possibility that early developmental influence (associated with a long-term modulation in androgen/estrogen production) plays a role in priming the ischemic stress response of the adult ArKO mouse heart. Levels of systemic estrogen are considerably reduced in ovariectomized animals, but a primary source of testosterone has also been removed. However, a significant amount of testosterone is still produced in the adrenal glands, and evidence suggests that tissue-specific aromatase expression increases significantly in ovariectomized animals through an apparent estrogen-dependent negative feedback action (56). Local production of estrogen may be maintained in the myocardium of ovariectomized animals. Poor recovery in ovariectomized animals can be reversed with dihydrotestosterone supplementation, restoring ventricular function to control level (14). The ArKO mouse has high plasma testosterone levels but no capacity to produce estrogen at a local (or ovarian) level. Further studies are required to confirm whether changes in estrogen production at the local, cardiac level are responsible for the modulated ischemic stress response observed in ArKO hearts and how the balance of androgen-to-estrogen impacts on the capacity to recover from an ischemic event.

In summary, using the ArKO model, this study demonstrates that aromatase deficiency improves cardiac postischemic functional performance and limits the extent of acute cardiomyocyte injury in early reperfusion. These findings challenge the notion of estrogen cardioprotection in ischemia/reperfusion and suggest that cardiac inotropic support may be facilitated by an increase in the relative level of testosterone to estrogen. The improved functional recovery in the ArKO was, however, associated with increased propensity for reperfusion-induced arrhythmia, indicative of modified cardiomyocyte Ca^{2+} handling. Thus, in specific contexts, estrogen withdrawal/suppression associated with testosterone elevation could promote more robust mechanical recovery after ischemia/reperfusion, but the potential benefit of this intervention would require coincident management of arrhythmogenic vulnerability.

Acknowledgments

We thank the generous gift of the Ar677 antibody from Novartis Pharma AG.

Address all correspondence and requests for reprints to: Lea M.D. Delbridge, Cardiac Phenomics Laboratory, Department of Physiology, University of Melbourne, Victoria 3010, Australia. E-mail: lmd@unimelb.edu.au.

This work was supported by the National Heart Foundation of Australia.

Disclosure Summary: The authors have nothing to disclose.

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