Aromatase transgenic upregulation modulates basal cardiac performance and the response to ischemic stress in male mice

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Bell JR, Bernasochi GB, Varma U, Boon WC, Ellem SJ, Risbridger GP, Delbridge LM. Aromatase transgenic upregulation modulates basal cardiac performance and the response to ischemic stress in male mice. Am J Physiol Heart Circ Physiol 306:H1265–H1274, 2014. First published March 7, 2014; doi:10.1152/ajpheart.00012.2014.—Estrogen in females is conventionally considered a cardioprotective influence, but a role for estrogen in male cardioprotection has yet to be defined. Estrogen biosynthesis from testosterone is regulated by aromatase. Aromatase has recently been shown to be expressed in the adult heart, although little is known about its involvement in the regulation of myocardial function and stress responses. The goal of this study was to determine whether upregulation of tissue aromatase expression could improve ischemic resilience in male hearts. Isolated hearts from male transgenic aromatase-overexpressing (AROM+) high estrogen, low testosterone (WT) male mice (12 wk) were Langendorff perfused and subjected to ischemia-reperfusion (25 min ischemia and 60 min of reperfusion). Basal systolic function was lower in AROM+ hearts (dP/dtmax: 4,121 ± 255 vs. 4,992 ± 283 mmHg/s, P < 0.05) and associated with augmented Akt phosphorylation, consistent with a suppressor action of estrogen on contractility. Ischemic contracture was attenuated in AROM+ hearts (43 ± 3 vs. 55 ± 4 mmHg, P < 0.05), yet AROM+ hearts were more arrhythmic in early reperfusion. At the end of 60 min of reperfusion, AROM+ systolic functional recovery was lower (left ventricular developed pressure: 39 ± 6 vs. 56 ± 5 %basal, P < 0.05) and diastolic dysfunction was accentuated (36 ± 4 vs. 24 ± 2 mmHg, P < 0.05). This is the first study to show that in vivo aromatase upregulation modulates basal cardiac performance and the response to ischemic stress. These data suggest that while chronic exposure to enhanced estrogenic influence may have benefits in limiting ischemic contracture severity, acute functional recovery in reperfusion is compromised. A temporally targeted, tissue-specific intervention combining aromatase treatment with inotropic support may offer therapeutic potential for men and women.

aromatase; ischemia/reperfusion; estrogen; contractile function; arrhythmia

CARDIOVASCULAR DISEASE is the major cause of death for both men and women in Westernized societies. Ischemic heart disease comprises a substantial component of this cardiovascular risk for both sexes, although onset of disease generally occurs 10 yr earlier in men (49). This apparent cardioprotection afforded to premenopausal women has contributed to the conventional view that estrogen is beneficial for the heart, yet controversy remains regarding the relative health benefits associated with estrogen hormone replacement therapy in postmenopausal women (50). A more complete mechanistic understanding of the actions of sex steroids on myocardial stress responses at a fundamental mechanistic level is required.

Experimentally, in numerous studies, female hearts have been shown to be more resilient to an acute ischemic insult compared with male control hearts (6). Female ex vivo hearts exhibit greater postischemic contractile recovery, fewer arrhythmias, and less necrotic/apoptotic death (8, 11, 36), which may at least partly be related to an improved capacity to manage high cellular Ca2+, an important mediator of ischemia-reperfusion pathologies. Acute ischemic protection in females is accentuated in models associated with high cellular Ca2+ loads (15, 16, 25). This may be related to fundamental differences in Ca2+ handling in male and female hearts. Specifically, it has been reported that increases in cytosolic Ca2+ are blunted in isolated female cardiomyocytes challenged with high extracellular Ca2+ (19), suggesting that female cardiomyocytes are adapted to maintain low operational Ca2+ levels even in high-Ca2+ stress conditions. The acute ischemic recovery advantage in females has been attributed to the actions of estrogen, as the reduced ischemic resilience observed in ovariectomized animals can be restored with estrogen supplementation (31, 34, 58). Whether cardiac-directed augmentation of estrogen has potential benefit has not been established, and the possibility of a beneficial role for estrogen in remediating ischemic stress in the male myocardium has been minimally investigated.

Estrogen biosynthesis is dependent on testosterone availability, with the cytochrome P-450 enzyme aromatase regulating the conversion of testosterone to estrogen. Aromatase is expressed in many extragonadal tissues, including bone, adipose, the prostate, and the brain, where it exerts important local actions within the milieu of fluctuating circulating hormone levels (51). We have recently shown that aromatase is expressed in the adult heart (7), where it has been localized to the coronary vasculature and to the myocardial tissue (32), extending earlier in vitro observations involving cultured neonatal myocytes (26). These reports have indicated that local estrogen
production in the heart may occur, although no measurements of basal aromatase activity in the myocardium have been reported to date. Little is known about the role of local steroid interconversion in the regulation of cardiac physiological function and stress responses. Modulation of aromatase activity secondary to changes in Ca\textsuperscript{2+} levels and signaling kinase pathways in other tissues (14) would indicate that aromatase has important influences on the myocardium in health and disease. Aromatase polymorphisms are linked to the risk of death in acute coronary syndrome in male patients (1). This intrinsic capability for local steroid conversion in the heart may assume particular importance when systemic estrogen levels are relatively low. Interventional capacity to specifically modulate cardiac aromatase expression to increase local estrogen levels in men and women is a feasible therapeutic goal as tissue-specific aromatase transcription-regulating genes have been described in various tissues [although they have not yet identified in the heart (51)]. A tissue-directed approach would obviate the undesirable effects in both men and women of systemic estrogen supplementation. Thus, while the case for the importance of intracardiac aromatase activity is apparent, at present the knowledge level is very modest. As a first step in understanding the potential of cardiac aromatase upregulation as a therapeutic target for augmenting tissue estrogen levels in men with ischemic heart disease, in the present study, we have assessed ex vivo ischemic resilience in a transgenic aromatase-overexpressing (AROM\textsuperscript{+}) mouse model.

METHODS

Animals. AROM\textsuperscript{+} mice were generated using a purified expression vector for human P-450 aromatase (pUbC-AROM) constructed using the pRC/CMV plasmid (Stratagene, La Jolla, CA) as a backbone with the cytomegalovirus promoter of the vector subsequently replaced with a 1.0-kb ubiquitin C promoter and raised on an FVB/N background, as previously described (37). AROM\textsuperscript{−} mice overexpress aromatase in the brain, testis, heart, and liver (32). Littermate AROM\textsuperscript{−} mice were designated as wild-type (WT) control mice. All mice were age matched (~12 and 40 wk) and maintained under identical conditions at the Biological Research Facility at the University of Melbourne (Melbourne, VIC, Australia). We have previously reported plasma sex steroid levels in male WT and AROM\textsuperscript{+} mice [see Ellem et al. (21)]. Male AROM\textsuperscript{−} mice exhibited low testosterone (~10-fold lower in AROM\textsuperscript{−} vs. WT mice at 12~–15 wk) and high relative levels of estrogen (~6-fold increase) (21). Experiments were conducted on mice handled in the manner specified by the Prevention of Cruelty to Animals Act 1986 and the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997), with approval and oversight of the Animal Ethics Committee of the University of Melbourne.

Isolated heart preparation. Isolated hearts were aerobiocically perfused, and heart function was monitored as previously described (5). Briefly, male and female mice were anesthetized [pentobarbital sodium (200 mg/kg ip)] in combination with sodium heparin (200 IU/kg ip), and hearts were rapidly excised and placed in cold (4°C) bicarbonate buffer. Hearts were then perfused in the noncirculating Langendorff mode with oxygenated (95% O\textsubscript{2}–5% CO\textsubscript{2}) bicarbonate buffer (37°C, pH 7.4) at a constant pressure equivalent to 80 mmHg (STH pump controller, AD Instruments, Bella Vista, NSW, Australia) and paced at 580 beats/min. Bicarbonate buffer contained (in mM) 119 NaCl, 4.0 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 22.0 NaHCO\textsubscript{3}, 1.2 MgCl\textsubscript{2}, 2.5 CaCl\textsubscript{2}, 0.5 EDTA, 2.0 Na pyruvate, and 5.0 glucose.

Hearts were aerobiocically perfused for 30 min before 25 min of global ischemia (37.0°C) and 60 min of reperfusion. Hearts were paced throughout the stabilization period and for the first 2 min of ischemia. Pacing subsequently recommenced at 5 min of reperfusion. Left ventricular (LV) pressure measurements were performed using a fluid-filled balloon connected to a pressure transducer (MLT8844) and recorded on a MacLab data-acquisition system (AD Instruments). The balloon was inflated to produce an end-diastolic pressure of 5–9 mmHg, and the volume was kept constant throughout the perfusion protocol. The parameters measured included LV developed pressure, heart rate, LV end-diastolic pressure (LVEDP), and maximal and minimal rates of change of ventricular pressure (\(dP/dt_{\text{max}}\) and \(dP/dt_{\text{min}}\), respectively). LV pressure traces were analyzed for the incidence and duration of ventricular fibrillation, as previously characterized against electrocardiogram tracings (30). Briefly, ventricular fibrillation was identified as a barely discernable beat of <5 mmHg. Arrhythmic activity was scored in a blinded manner for the first 5 min of reperfusion. At the end of reperfusion, ventricles were snap frozen in liquid nitrogen for molecular analyses.

Immunoblot analysis. LV tissue from nonperfused and perfused hearts was homogenized [20 mmol/l Tris·HCl (pH 6.8), 5 mmol/l EGTA, 5 mmol/l EDTA, 5 mmol/l NaF, 0.5 mmol/l Na\textsubscript{3}VO\textsubscript{4} + protease inhibitor cocktail (Complete, Roche), 4°C, 10% (wt/vol) with a Polytron tissue grinder and reconstituted in equal volumes of 2× SDS sample buffer (4). Ventricular sample protein concentrations were determined using a modified Lowry assay (43). Equal amounts of protein were loaded onto polyacrylamide gels (10~–15%) for SDS-PAGE and Western blot analysis. The primary antibodies used include sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase 2a (SERCA2a; Affinity Bioreagents), total phospholamban (PLB; Upstate), phosphorylated (p)-PLB (Thr\textsuperscript{17}, Badrilla, Leeds, UK), total Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII; gift from Donald Bers, University of California, Davis, CA), autophosphorylated CaMKII [p-CaMKII (Thr\textsuperscript{287}); Abcam], total p38 MAPK, p-p38MAPK (Thr\textsuperscript{180}/Tyr\textsuperscript{182}), Akt, p-Akt (Ser\textsuperscript{473}), p-Akt (Ser\textsuperscript{320}), Bax1, Bcl-2, LC3BII, and beclin-1 (all Cell Signalling). Protein bands were visualized with a Bio-Rad Chemi-XRS imaging device, and band intensity was quantified using Quantity One imaging software (Bio-Rad). Equal loading of cardiac homogenate samples was verified by subsequent densitometric scanning of Coomassie-stained membranes.

Statistical analysis. Results are presented as means ± SE. Differences between groups were assessed with a Student’s unpaired t-test, one-way ANOVA with repeated measures, or two-way ANOVA with Fisher’s least-significant-difference post hoc analysis as appropriate. Differences were considered significant at P < 0.05. All statistical calculations were performed using SPSS (version 13.0, SPSS, Chicago, IL).

RESULTS

Lower body weight and heart growth in AROM\textsuperscript{+} mice. The mean body weight of male 12-wk-old AROM\textsuperscript{+} mice was slightly but significantly lower (by 10%) than WT control mice (Table 1), in accordance with previous observations (38). This lower somatic weight was associated with a nonsignificant trend toward a reduction in ventricular weight (combined LV and right ventricle), and no difference in the ventricular-to-body weight index was thus observed. Tibia length was not used as a marker of mouse size for cardiac weight normalization, as aromatase has well-established influences on bone density/growth (53).

Lower basal contractile function associated with increased Akt activity. Basal contractile performance was assessed in isolated Langendorff-perfused hearts from 12-wk-old WT and AROM\textsuperscript{+} mice (Table 1). After a 30-min stabilization period, AROM\textsuperscript{+} hearts exhibited significantly lower contractile func-
tion compared with WT control hearts. This differential was modest (~14%) and most evident in systolic function, with \(\text{dP/dt}_{\text{max}}\) significantly lower in AROM\(^{+}\) hearts, and there was a similar nonsignificant trend for a lower LV developed pressure. Coronary flow (normalized to heart weight) was similar in AROM\(^{+}\) and WT mice. These ex vivo findings are consistent with the hypocontractile influence of estrogen previously reported in isolated cardiomyocytes (18).

To evaluate the mechanisms contributing to this relatively suppressed basal contractile function in AROM\(^{+}\) hearts, the expression levels of relevant proteins involved in excitation-contraction coupling were assessed in nonperfused normoxic hearts by Western blot analysis. As shown in Fig. 1, no differences in the total expression of selected Ca\(^{2+}\)-handling proteins (including SERCA2a, PLB, and CaMII) or signaling intermediates implicated in sex steroid signaling (p38 MAPK and Akt) were detected between groups. However, the AROM\(^{+}\) myocardium did exhibit a marked and significantly higher (80%) of Akt phosphorylation (1.41 ± 0.16 vs. 0.88 ± 0.06 arbitrary units, \(P < 0.05\)). No differences in the phosphorylation states of p38 MAPK, PLB, or CaMII were observed.

Suppressed ischemic contracture in the AROM\(^{+}\) mouse heart. AROM\(^{+}\) and WT hearts subjected to 25 min of global ischemia exhibited pronounced ischemic contracture, as shown in the representative traces throughout ischemia-reperfusion in Fig. 2A. The onset and amplitude of contracture were measured as indexes of injury sustained during the ischemic period. Figure 2B shows that contracture generally occurred later and to a lesser extent in AROM\(^{+}\) hearts compared with WT hearts (amplitude: 43 ± 3 vs. 55 ± 4 mmHg, \(n = 7–8, P < 0.05\)). This indicates that AROM\(^{+}\) hearts were less susceptible to ATP depletion and cellular Ca\(^{2+}\) accumulation in ischemia (29), exhibiting less prominent symptoms of rigor- and/or hypercontracture-associated myocyte rupture.

Poor functional outcomes in reperfused AROM\(^{+}\) mouse hearts. Despite the indication from ischemic contracture data that AROM\(^{+}\) hearts were more resistant to the progression of ischemic injury, these hearts surprisingly exhibited diminished and unstable functional performance in reperfusion. As shown in Fig. 3, AROM\(^{+}\) hearts were more arrhythmic in the initial 5 min after ischemia, with a twofold greater total duration of ventricular fibrillation (AROM\(^{+}\) vs. WT hearts: 194 ± 12 vs. 103 ± 22 s, \(n = 7–8, P < 0.05\)). Furthermore, the recovery of systolic contractile function was lower throughout 60 min of reperfusion (Fig. 4). The recovery (at 60 min of reperfusion, as %basal) of both LV developed pressure (39 ± 6 vs. 56 ± 6 %basal, \(n = 7–8, P < 0.05\)) and \(\text{dP/dt}_{\text{max}}\) (44 ± 7 vs. 68 ± 7 %basal, \(n = 7–8, P < 0.05\)) was reduced in AROM\(^{+}\) hearts. Taking into consideration the initially lower basal function of AROM\(^{+}\) hearts, this recovery deficit comprised a substantial loss of contractile capacity in the setting of ischemic stress. Additionally, the recovery of diastolic relaxation after ischemia was compromised in AROM\(^{+}\) hearts, as evidenced by a high LVEDP (36 ± 4 vs. 24 ± 4 mmHg, \(n = 7–8, P < 0.05\)) and low recovery of \(\text{dP/dt}_{\text{min}}\) (39 ± 5 vs. 57 ± 5 %basal, \(n = 7–8, P < 0.05\)) after 60 min of reperfusion.

This contractile dysfunction in reperfused AROM\(^{+}\) hearts was not associated with modulated susceptibility to apoptosis or autophagy, as assessed in hearts snap frozen at the end of 60 min of reperfusion (Fig. 5). Western blot analysis showed that the ratio of Bax to Bcl-2, a marker of apoptotic cell death, was similar in AROM\(^{+}\) and WT hearts. Similarly, beclin-1 expression and the ratio of LC3BII to LC3BI, both indicative of autophagosome formation, were not different in AROM\(^{+}\) and WT hearts.

Diminished effect on ischemia-reperfusion function in female AROM\(^{+}\) hearts. For comparative purposes, the genotype differences in male cardiac responses were referenced to female performance outcomes. No differences was observed in responses of male and female WT hearts to ischemia and reperfusion (Fig. 6, A–D), as previously reported (2). A sex-genotype interaction was observed in the ischemic contracture amplitude (amplitude: male AROM\(^{+}\) vs. WT mice, 43 ± 3 vs. 55 ± 4 mmHg; female AROM\(^{+}\) vs. WT mice, 61 ± 8 vs. 48 ± 6 mmHg, means ± SE, \(n = 6–8\) hearts/group, sex × genotype interaction, two-way ANOVA with least-significant-difference post hoc analysis; Fig. 6A). Contractile function in ischemia was not different between female AROM\(^{+}\) and WT mice. Reperfusion parameters were also not different between female genotypes, and, in general, these were most similar to male WT performance levels, suggesting that the effect of an augmented androgen-estrogen conversion capacity is more marked in males than in females.

Reperfusion dysfunction is abrogated in aged AROM\(^{+}\) mouse hearts. As previously documented, AROM\(^{+}\) mice are characterized by high estrogen and low testosterone systemic levels (21) and, thus, at 12 wk of age, exhibit very low testosterone-to-estrogen ratios compared with WT control mice. In male WT mice, the testosterone-to-estrogen ratio
**Fig. 1.** Sex steroid signaling and Ca\(^{2+}\)-handling protein expression and phosphorylation status in normoxic wild-type (WT) and transgenic aromatase-overexpressing (AROM\(^+\)) hearts. 

**A**: AROM\(^+\) hearts exhibited a significant increase in phosphorylated (P-)Akt levels, and no differences were observed in total Akt expression or the ratio of P-Akt to Akt.

**B**: No differences were observed in the expression of p38 MAPK or P-p38 MAPK.

**C**: Expression of sarcoplasmic reticulum Ca\(^{2+}\)-handling proteins sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), phospholamban (PLB), and P-PLB were not different.

**D**: Similarly, no differences were observed in Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) expression or phosphorylation. Representative blots for CaMKII \(\delta_b\) and \(\delta_c\) are duplicated, with \(\delta_b\) (top band) and \(\delta_c\) (bottom band) band intensity quantified separately. Values are means ± SE; \(n = 6–8\) hearts/group (age: 12 wk). *P < 0.05 by Student’s t-test.
hearts/group (age: 12 wk). In ischemia, the relative diminution of contracture observed in 12-wk-old AROM WT control mice; not shown). In ischemia, the relative diminution of contracture was lower in AROM 12-wk-old mice, hearts from 40-wk-old male WT and AROM 12-wk values for comparison in Fig. 7. No differences were observed in baseline function in 40-wk-old AROM mice, a characteristic that likely reflects intrinsic downregulation of cardiomyocyte performance under a proestrogenic influence (18, 19). Ischemic contracture is suppressed in AROM hearts, yet a paradoxical increase in arrhythmogenic activity is observed in early reperfusion. Furthermore, reperfusion recovery (relative to preischemic basal levels) of both
developed pressure at 60 min of reperfusion compared with younger WT hearts, although this did not reach statistical significance (40 wks: 40 ± 5 mmHg vs. 12 wk: 56 ± 7 mmHg, n = 6–8, P = 0.09). In aged AROM hearts, the recovery of systolic function (i.e., LV developed pressure) and diastolic function (i.e., LVEDP) parameters was not significantly different at 60 min of reperfusion relative to age-matched WT hearts (Fig. 7, C and D). These observations indicate that with the less marked aromatase-mediated shift in the endogenous testosterone-to-estrogen ratio in aged AROM mice (relative to WT mice), the alterations in cardiac contractile performance exhibited by younger animals under normoxic, ischemic, and reperfusion conditions were mitigated.

DISCUSSION

This is the first study to show that chronic in vivo genetic upregulation of tissue aromatase in a murine model modulates basal myocardial contractile function and intracellular signaling and has detrimental effects on the acute ex vivo cardiac recovery response to an ischemic stress event in male animals. In the AROM mouse, systemic and tissue levels of estrogen are elevated and testosterone levels are reciprocally diminished, with the testosterone-to-estrogen ratio markedly reduced. In mature young male mice, the basal level of cardiac contractility is significantly lower in AROM compared with WT mice, a characteristic that likely reflects intrinsic downregulation of cardiomyocyte performance under a proestrogenic influence (18, 19). Ischemic contracture is suppressed in AROM hearts, yet a paradoxical increase in arrhythmogenic activity is observed in early reperfusion. Furthermore, reperfusion recovery (relative to preischemic basal levels) of both declines with age, and, with aging, the relative difference in this ratio between genotypes progressively declines. By the age of 40 wk, the difference in the testosterone-to-estrogen ratio between AROM and WT mice is half the value at 12 wk (21). Thus, in a parallel set of experiments, we assessed how comparative ischemia-reperfusion vulnerability between AROM and WT mice was modulated under conditions of less pronounced proestrogenic status. Similar to the experiments in 12-wk-old mice, hearts from 40-wk-old male WT and AROM mice were isolated and subjected to an ischemia-reperfusion challenge. Data are shown with the inclusion of reference 12-wk values for comparison in Fig. 7. No differences were observed in basal function in 40-wk-old AROM mice (vs. WT control mice; not shown). In ischemia, the relative diminution of the contracture observed in 12-wk-old AROM mice was not discernible at the age of 40 wk (Fig. 7A). However, 40-wk-old AROM mice remained susceptible to arrhythmogenicity in reperfusion, exhibiting ventricular fibrillation activity at levels observed in younger 12-wk-old mice (40 wk: 161 ± 32 vs. 66 ± 13 s, n = 6–8, P < 0.05; Fig. 7B). Aged WT hearts exhibited a relative reduction in the recovery of LV

Fig. 2. Ischemic contracture in WT and AROM hearts. A: representative pressure records. B: the development of contracture occurred later in AROM hearts during 25 min ischemia. Values are means ± SE; n = 7–8 hearts/group. *P < 0.05 by ANOVA with repeated measures. C: the maximal amplitude of contracture was lower in AROM hearts. Values are means ± SE; n = 7–8 hearts/group (age: 12 wk). *P < 0.05 by Student’s t-test.

Fig. 3. Severity of ventricular fibrillation in WT and AROM hearts in the first 5 min of reperfusion. A: representative traces of left ventricular (LV) pressure in hearts in sinus rhythm and ventricular fibrillation. B: the total duration of ventricular fibrillation during the first 5 min of reperfusion was greater in AROM hearts. Values are means ± SE; n = 7–8 hearts/group (age: 12 wk). *P < 0.05 by Student’s t-test.
systolic and diastolic function is less robust in AROM hearts, suggesting that the combination of low testosterone and high estrogen levels may not provide the necessary inotropic support required to maximize contractile function in the acute postischemic stress period. Collectively, these data demonstrate a role for aromatase in the modulation of cardiac function basally and in response to ischemia-reperfusion in males. In females, a minimal impact of transgenic aromatase overexpression was observed. These findings suggest that targeted and timely cardiac interventions to manipulate relative androgen and estrogen levels have the potential to minimize ischemic damage and maximize reperfusion recovery.

Basal functional implications of aromatase overexpression.

The finding that a global overexpression of aromatase in males, which exhibit a phenotype of high systemic estrogen with low testosterone levels, resulted in a diminution of basal isolated heart contractility function (Table 1) was predicted and consistent with previous reports describing estrogen suppression and testosterone augmentation of ex vivo heart contractility (47, 48, 52). Gonadectomy studies have shown that these sex differences are strongly influenced by systemic sex steroid levels (17, 18). We and others have previously shown that the contractile performance of single isolated male cardiomyocytes is more vigorous than female myocytes when measured under standardized conditions and that operational myocyte Ca\(^2\)\(^+\) levels are higher in male cells (19, 24, 45). Thus, contractile modulation at myocyte and intact heart levels show concordant influences. Interestingly, it has been demonstrated that the

![Fig. 4. Recovery of contractile function throughout 60 min of reperfusion. A: percentage recovery of systolic function at the end of 60 min of reperfusion, relative to the basal preischemic value, was lower in AROM hearts, both in terms of LV developed pressure (DevP) and dP/dt\(_{\text{max}}\). B: diastolic dysfunction was also exacerbated in AROM hearts, with increased LV end-diastolic pressure (LVEDP) and reduced recovery of dP/dt\(_{\text{min}}\) at the end of reperfusion. Values are means ± SE; n = 7–8 hearts/group (age: 12 wk). *P < 0.05 by Student’s t-test.](image)

![Fig. 5. Immunoblot analysis of programmed cell death at the end of 60 min of reperfusion. A: the ratio of Bax to Bcl-2 was assessed as a marker of apoptosis and found not to differ between WT and AROM hearts. B: no genotypic differences were observed in the ratio of LC3BI to LC3BII, which is representative of the formation of autophagosomes. C: similarly, beclin-1 expression was unchanged in WT and AROM hearts. Values are means ± SE; n = 7–8 hearts/group (age: 12 wk). P = not significant by Student’s t-test.](image)
distribution and expression of androgen and estrogen receptor subtypes are not sex dependent in the mouse heart (41).

The cellular mechanisms responsible for the actions of sex and sex steroids on contractility remain poorly understood and are likely multifaceted. Expression effects on myocardial Ca\(^{2+}\)-handling proteins have been reported, although there is a considerable discrepancy in the literature (2, 45). We did not observe any differences in the expression and/or CaMKII-specific phosphorylation status of sarcoplasmic reticulum Ca\(^{2+}\) reuptake proteins (PLB and SERCA2a; Fig. 1) between AROM\(^{+}\) and WT mice, which is consistent with the lack of difference in basal diastolic function. The phosphorylation status of Akt was increased in AROM\(^{+}\) hearts, and this finding is consistent with attenuated contractility in these hearts. The elevated estrogen levels in AROM\(^{+}\) is consistent with attenuated contractility in these hearts. The phosphorylation status of Akt was increased in AROM\(^{+}\) hearts, and this finding is consistent with attenuated contractility in these hearts. The elevated estrogen levels in AROM\(^{+}\) animals (21) likely facilitate Akt stimulation (13). Evidence shows that nongenomic specific phosphorylation status of sarcoplasmic reticulum Ca\(^{2+}\) are widespread (9), and its ablation has been linked with reduced Ca\(^{2+}\) entry currents and expression (42, 54), possibly involving nitrosylation.

Reperfusion dysfunction in AROM\(^{+}\) hearts despite indicators of less severe ischemic damage. During the ischemic period, a delayed onset and/or lower-amplitude contracture is generally considered predictive of favorable posts ischemic outcomes (29). Hence, the suppressed ischemic contracture in male AROM\(^{+}\) hearts (Fig. 2) would be consistent with myocardial salvage actions attributed to estrogen. However, contractile function in AROM\(^{+}\) mice was diminished in reperfusion, with accentuated arrhythmogenicity and impaired reperfusion contractile recovery (Figs. 3 and 4). As an in vivo model of high estrogen, this adverse finding in AROM\(^{+}\) mice is entirely unexpected and challenges the current dogma regarding the relative benefits associated with cardiac estrogen and testosterone exposure. An attribution of the distinctive cardiac basal and reperfusion responses of AROM\(^{+}\) animals to an altered androgen-estrogen balance is supported by the findings in aged animals. We have previously reported that the proestrogenic status of male AROM\(^{+}\) mice declined with age, and, thus, we used 40-wk-old male mouse hearts as a model to further determine how an altered androgen-estrogen balance influenced the ischemic stress response. At 40 wk, when the testosterone-to-estrogen ratio difference between AROM\(^{+}\) and WT mice was markedly reduced, the cardiac systolic and diastolic functional phenotypes were also essentially abrogated (although an arrhythmogenic disposition persisted). Aging may play a factor in the response to ischemia-reperfusion challenge in WT and AROM\(^{+}\) hearts (28). This suggests that the functional response to ischemia-reperfusion may be influenced by the relative levels of circulating/local testosterone and estrogen and indicates that the ischemic stress response may change in men and women as their androgen/estrogen status changes with age.

Extensive (but not unanimous) experimental literature supports the notion of a beneficial role for estrogen in modulating impacts of ischemia-reperfusion (56), although estrogen supplementation can have sexually dimorphic actions and may be
a liability in males in certain myocardial disease settings (27). This is consistent with data presented in the present study, where the high estrogen/low testosterone levels in AROM+/H11001 male hearts had a detrimental effect on postischemic functional recovery relative to both WT and female AROM+/H11001 hearts. Very recent evidence in experimental models of ischemic stroke has indicated that estrogenic actions on the G protein-coupled estrogen receptor can have opposing actions on injury in males and females (10), but a similar relationship has not been shown in the heart (20). These studies do suggest that sex steroids actions are complex and the extent to which they exert beneficial/detrimental actions may be context specific. Indeed, for premenopausal women (relative estrogen replete state), myocardial infarction is associated with a greater vulnerability to heart failure development and an increased mortality risk (55). The increased duration of ventricular fibrillation in male AROM+/H11001 mice was surprising, given the lower incidence of sudden cardiac death in women. It is not clear why a high estrogen/low testosterone state would increase the vulnerability to arrhythmias, or why this remained high at 40 wk, when the sex steroid ratio in AROM+/H11001 animals was less different compared with WT animals. The arrhythmias reported in AROM+/H11001 and WT hearts at 40 wk were indirectly inferred from mechanical measurements as previously validated (30). Future detailed electrocardiographic work may provide more insights into the origins and mechanisms of these irregular beats. Acute estrogen treatment has been shown to reduce arrhythmias in male hearts, although to a lesser extent than that reported in female hearts (46). Conversely, there is evidence showing that testosterone can also reduce the extent of fatal arrhythmias (35), although the mechanisms responsible have not been studied. The influence of sex steroids on cardiac reperfusion arrhythmias represents the outcome of multiple and complex actions on specific ion channel expression/activity, membrane excitability, and electrical conduction, which are all profoundly modulated in myocardial infarction.

In contrast to estrogen findings, emerging evidence suggests a beneficial role for testosterone in ischemia-reperfusion. Findings from castration models have been equivocal, with postischemic functional recovery observed to be reduced, increased, or unaffected (33, 40, 44). Nonaromatisable testosterone (dihydrotestosterone) supplementation has been shown to improve recovery in reperfusion in castrated males (12) and promote myocardial viability in ovariectomized female hearts subjected to ischemia-reperfusion to an extent that is similar to estradiol supplementation alone (39). The mechanisms for these effects of androgens have not been resolved. Activation of signaling intermediates associated with cardioprotective preconditioning mechanisms, including mitochondrial ATP-dependent K+/H11006 channels and heat shock protein 70, have been implicated (22, 40). Although activation of these mediators would be expected to suppress the induction of cell death signaling during ischemia-reperfusion, no differences between AROM+/H11001 and WT animals in the expression of cell death markers (apoptotic and autophagic) were observable at the end of reperfusion (Fig. 5). If unrelated to differences in cell viability signaling, the diminished recovery of AROM+/H11001 hearts
in reperfusion may alternatively imply an inadequacy of testosterone-mediated inotropic support.

Aromatase: a potential cardiac intervention target in mediating sex steroid cardiac support? With the recent demonstration that aromatase is expressed in the mature adult myocardium (6), the potential for cardiac-specific androgen-estrogen conversion (superimposed on variable levels of circulating steroid levels) arises. This study provides evidence showing that in male rodents, while chronic estrogen elevation (through means of genetic aromatase augmentation) appears to suppress the ischemic injury response, it does not confer immediate postischemic functional resilience, a finding not consistent with the general notion of estrogen protection. The development of a cardiac-specific aromatase expression model will provide more insights into local aromatase actions in a setting not limited by secondary systemic effects of endocrine disturbance. Other experimental indications of aromatase-mediated cardiac action in the acute setting of an ischemic episode have recently emerged. In a murine model of aromatase deficiency (high testosterone/low estrogen), immediate postischemic functional rebound is enhanced in females (7). Pharmacological intervention with an aromatase inhibitor before experimental coronary artery occlusion in male animals improves tissue salvage (32).

These effects suggest that intracardiac steroid conversion during the progression of an acute event occurs, which could be ascribed to the reciprocal regulation of the testosterone/ estrogen balance (rather than attributed to altered estrogen alone). An approach involving estrogen maximization during ischemia and minimization in the early reperfusion window (with reciprocal testosterone action) may be therapeutically valid in both males and females. This study provides the impetus for exploration of the potential for cardiac-specific aromatase modulation of sex steroid levels using tissue-specific delivery modes. Given the tested clinical acceptance of aromatase inhibitors used in other disease settings, there is a promising translational scope for targeting aromatase to optimize ischemic cardiac outcomes by achieving a testosterone-estrogen balance. A temporally defined, tissue-specific intervention combining aromatase treatment with inotropic support and arrhythmic management may offer therapeutic potential for men and women.

GRANTS
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AUTHOR CONTRIBUTIONS

REFERENCES


