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

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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 posts ischemic 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\(^{2+}\) 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\(^{+}\)) mouse model.

**METHODS**

**Animals.** AROM\(^{+}\) 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\(^{+}\) mice overexpress aromatase in the brain, testis, heart, and liver (32). Littermate AROM\(^{-}\) 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\(^{+}\) mice [see Ellem et al. (21)]. Male AROM\(^{+}\) mice exhibited low testosterone (~10-fold lower in AROM\(^{+}\) 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 aerobic perfused and analyzed for basal contractile function, as aromatase has well-established influences on bone density/growth (53). Lower body weight and heart growth in AROM\(^{+}\) mice. The mean body weight of male 12-wk-old AROM\(^{+}\) 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\(^{+}\) mice (Table 1). After a 30-min stabilization period, AROM\(^{+}\) hearts exhibited significantly lower contractile func-
tion compared with WT control hearts. This differential was modest (~14%) and most evident in systolic function, with dP/dt 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 Ca2+-handling proteins (including SERCA2a, PLB, and CaMKII) 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 level (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 CaMKII 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 Ca2+ 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 dP/dt 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 dP/dt 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 more 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

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**Table 1. Animal and heart weight characteristics and basal ex vivo cardiac performance in male 12- and 40-wk-old mice**

<table>
<thead>
<tr>
<th></th>
<th>12 wk</th>
<th>40 wk</th>
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<tbody>
<tr>
<td></td>
<td>WT mice</td>
<td>AROM+ mice</td>
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<tr>
<td></td>
<td>WT mice</td>
<td>AROM+ mice</td>
</tr>
<tr>
<td><strong>Morphology</strong></td>
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<tr>
<td>Body weight, g</td>
<td>31.2 ± 0.8</td>
<td>28.6 ± 0.7*</td>
</tr>
<tr>
<td>Ventricular weight, mg</td>
<td>190 ± 7</td>
<td>182 ± 5</td>
</tr>
<tr>
<td>Ventricular weight/body weight, mg/g</td>
<td>6.1 ± 0.2</td>
<td>6.4 ± 0.1</td>
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<tr>
<td><strong>Basal function</strong></td>
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<tr>
<td>Left ventricular developed pressure, mmHg</td>
<td>94 ± 5</td>
<td>81 ± 6</td>
</tr>
<tr>
<td>dP/dt max, mmHg/s</td>
<td>4,992 ± 283</td>
<td>4,121 ± 255*</td>
</tr>
<tr>
<td>dP/dt min, mmHg/s</td>
<td>−2,780 ± 111</td>
<td>−2,489 ± 120</td>
</tr>
<tr>
<td>Coronary flow, ml·min⁻¹·mg⁻¹</td>
<td>17.7 ± 0.4</td>
<td>17.0 ± 1.3</td>
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Data are presented as means ± SE; n = 7–8 mice/group. WT, wild type; AROM+, transgenic aromatase-overexpression; dP/dt max, maximal rate of pressure change (contraction); dP/dt min, maximal rate of pressure change (relaxation). *P < 0.05, AROM+ vs. WT mice.
Fig. 1. Sex steroid signaling and Ca\textsuperscript{2+}-handling protein expression and phosphorylation status in normoxic wild-type (WT) and transgenic aromatase-overexpressing (AROM\textsuperscript{+}) hearts. A: AROM\textsuperscript{+} 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\textsuperscript{2+}-handling proteins sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA), phospholamban (PLB), and P-PLB were not different. D: similarly, no differences were observed in Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) expression or phosphorylation. Representative blots for CaMKII\textsuperscript{B} and CaMKII\textsuperscript{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.
Aromatase modulates the cardiac ischemic stress response

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.

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
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.
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
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\(^+\) 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 steroid 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. Values are means ± SE; n = 6–8 hearts/group. *P < 0.05 by two-way ANOVA with LSD post hoc analysis.

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⁺ 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+ 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+ 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.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


