Flutamide restores cardiac function after trauma-hemorrhage via an estrogen-dependent pathway through upregulation of PGC-1

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Flutamide restores cardiac function after trauma-hemorrhage via an estrogen-dependent pathway through upregulation of PGC-1. Although previous studies have shown that flutamide improves cardiovascular function after trauma-hemorrhage, the mechanisms responsible for the salutary effect remain unknown. We hypothesized that flutamide mediates its beneficial effects via an estrogen-dependent pathway through upregulation of peroxisome proliferator-activated receptor-γ coactivator 1 (PGC-1). PGC-1, a key regulator of cardiac mitochondrial ATP production, induces mitochondrial DNA (mtDNA)-encoded genes such as cytochrome- cog oxidase (COX) subunit I, II, and III (COX I, COX II, and COX III), which regulates mitochondrial oxidative phosphorylation. To test this hypothesis, male rats underwent trauma-hemorrhage (mean arterial pressure of 35–40 mmHg for ~90 min) followed by resuscitation. At the onset of resuscitation, rats received vehicle, flutamide (25 mg/kg body wt), flutamide in combination with estrogen receptor (ER) antagonist ICI-182,780 (3 mg/kg body wt), or ICI-182,780 alone. Flutamide administration after trauma-hemorrhage restored the depressed cardiac function and increased cardiac testosterone, estrogen levels, and aromatase activity. These increases were accompanied by normalized cardiac ER-α and ER-β protein levels, PGC-1, and COX I mRNA expression, mitochondrial COX activity, and ATP contents. However, cardiac dihydrotestosterone, 5α-reductase II, androgen receptor protein levels, and mtDNA-encoded genes COX II and COX III were unaffected by flutamide treatment. The flutamide-mediated restoration of cardiac function, the increases in aromatase activity and estrogen levels, ER-α, ER-β, PGC-1, COX I, COX activity, and ATP contents were, however, abolished when ER antagonist ICI-182,780 was administered along with flutamide. These findings suggest that the salutary effect of flutamide on cardiac function after trauma-hemorrhage is mediated via an estrogen-dependent pathway through upregulation of PGC-1.

Despite numerous advances in intensive care medicine, ischemia-reperfusion injury, sepsis, and organ dysfunction leading to multiple organ failure remain the major causes of death in trauma patients as well as in patients after major surgery (4, 10, 11, 14, 30, 32, 36). Previous studies (2, 11, 13, 22) have shown that despite fluid resuscitation, there is a prolonged depression of cardiovascular function in males after trauma-hemorrhage. The depletion of androgen levels by castration prevented the depression in cardiac function after trauma-hemorrhage (21, 34). Moreover, administration of 17β-estradiol after trauma-hemorrhage restored the depressed cardiovascular function (9, 16). These studies, therefore, indicate that male and female sex steroids differentially influence cardiovascular function after trauma-hemorrhage. In this regard, flutamide, a nonsteroidal androgen receptor (AR) antagonist, has been shown to improve cardiac and immune function in male mice and rats after trauma-hemorrhage (22, 31, 35). Our previous study (24) has also demonstrated that pretreatment of males with flutamide increased estrogen receptor (ER) expression in T lymphocytes. Moreover, we have recently found that flutamide upregulates cardiomyocyte ER expression after trauma-hemorrhage (35). Although the previous study has shown that flutamide upregulates ER expression, it remains unclear whether flutamide mediates its salutary effects via upregulating estrogen-dependent pathway.

Studies have reported that flutamide increases plasma testosterone and estrogen levels (17, 19, 33). Testosterone is converted to estrogen and dihydrotestosterone (DHT) by the aromatase and 5α-reductase enzymes, respectively, and it is well known that estrogen and DHT interact with the specific receptors ER and AR (25, 29). Our previous studies have shown that estrogen administration after trauma-hemorrhage restored cardiac function. However, the salutary effects of estrogen were abolished if the ER antagonist ICI-182,780 was administered along with estrogen (9). In addition, peroxisome proliferator-activated receptor-γ coactivator 1 (PGC-1) is a regulator of cardiac mitochondrial ATP production, and it induces mitochondrial DNA (mtDNA)-encoded genes such as cytochrome-c oxidase (COX) subunit I, II, and III (COX I, COX II, and COX III), which are required for mitochondrial oxidative phosphorylation (12). Although we have found that PGC-1 upregulation appears to be one of the mechanisms by which flutamide mediates its salutary effects on cardiac function after trauma-hemorrhage (6), it remains unclear whether the effect of flutamide on PGC-1 downstream effects is mediated via ER. We hypothesized that flutamide mediates its beneficial effects via estrogen-dependent pathway through upregulation of PGC-1 and its target genes. To test this hypothesis, the effect of flutamide either alone or in combinations with the ER antagonist ICI-182,780 in rats after trauma-hemorrhage was examined on cardiac 1) function; 2) sex steroid hormones; 3) steroidogenic enzymes; 4) ER-α, ER-β, and AR protein levels; 5) PGC-1 gene expression; 6) mtDNA-encoded COX I, COX II, COX III gene expressions and COX activity; and 7) mitochondrial ATP contents.

Am J Physiol Heart Circ Physiol 290: H416–H423, 2006. First published September 9, 2005; doi:10.1152/ajpheart.00865.2005.—Although the previous study has shown that flutamide upregulates cardiac and immune function after trauma-hemorrhage, the mechanisms responsible for the salutary effect remain unknown. We hypothesized that flutamide mediates its beneficial effects via an estrogen-dependent pathway through upregulation of peroxisome proliferator-activated receptor-γ coactivator 1 (PGC-1). PGC-1, a key regulator of cardiac mitochondrial ATP production, induces mitochondrial DNA (mtDNA)-encoded genes such as cytochrome-c oxidase (COX) subunit I, II, and III (COX I, COX II, and COX III), which regulates mitochondrial oxidative phosphorylation. To test this hypothesis, male rats underwent trauma-hemorrhage (mean arterial pressure of 35–40 mmHg for ~90 min) followed by resuscitation. At the onset of resuscitation, rats received vehicle, flutamide (25 mg/kg body wt), flutamide in combination with estrogen receptor (ER) antagonist ICI-182,780 (3 mg/kg body wt), or ICI-182,780 alone. Flutamide administration after trauma-hemorrhage restored the depressed cardiac function and increased cardiac testosterone, estrogen levels, and aromatase activity. These increases were accompanied by normalized cardiac ER-α and ER-β protein levels, PGC-1, and COX I mRNA expression, mitochondrial COX activity, and ATP contents. However, cardiac dihydrotestosterone, 5α-reductase II, androgen receptor protein levels, and mtDNA-encoded genes COX II and COX III were unaffected by flutamide treatment. The flutamide-mediated restoration of cardiac function, the increases in aromatase activity and estrogen levels, ER-α, ER-β, PGC-1, COX I, COX activity, and ATP contents were, however, abolished when ER antagonist ICI-182,780 was administered along with flutamide. These findings suggest that the salutary effect of flutamide on cardiac function after trauma-hemorrhage is mediated via an estrogen-dependent pathway through upregulation of PGC-1.

DESPITE NUMEROUS ADVANCES in intensive care medicine, ischemia-reperfusion injury, sepsis, and organ dysfunction leading to multiple organ failure remain the major causes of death in trauma patients as well as in patients after major surgery (4, 10, 11, 14, 30, 32, 36). Previous studies (2, 11, 13, 22) have shown that despite fluid resuscitation, there is a prolonged depression of cardiovascular function in males after trauma-hemorrhage. The depletion of androgen levels by castration prevented the depression in cardiac function after trauma-hemorrhage (21, 34). Moreover, administration of 17β-estradiol after trauma-hemorrhage restored the depressed cardiovascular function (9, 16). These studies, therefore, indicate that male and female sex steroids differentially influence cardiovascular function after trauma-hemorrhage. In this regard, flutamide, a nonsteroidal androgen receptor (AR) antagonist, has been shown to improve cardiac and immune function in male mice and rats after trauma-hemorrhage (22, 31, 35). Our previous study (24) has also demonstrated that pretreatment of males with flutamide increased estrogen receptor (ER) expression in T lymphocytes. Moreover, we have recently found that flutamide upregulates cardiomyocyte ER expression after trauma-hemorrhage (35). Although the previous study has shown that flutamide upregulates ER expression, it remains unclear whether flutamide mediates its salutary effects via upregulating estrogen-dependent pathway.

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MATERIALS AND METHODS

Rat trauma-hemorrhagic shock model. Male Sprague-Dawley rats (Charles River) were fasted overnight but were allowed free access to water before the experiments. This study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Trauma-hemorrhage and resuscitation were then carried out as described previously (2, 3, 7, 9, 21, 22, 34). Briefly, rats were anesthetized by isoflurane inhalation, and a 5-cm midline laparotomy was performed to induce soft tissue trauma. The abdominal wound was then closed in layers, and polyethylene catheters (PE-50, Becton Dickinson, Sparks, MD) were placed in both femoral arteries and the right femoral vein and subsequently tunneled through to the dorsal surgical and the incision sites closed. The rats were then placed into a Plexiglas box (21 × 9 × 5 cm) in a prone position and allowed to awaken, after which they were bled rapidly within 10 min to a mean arterial pressure (MAP) of 35–40 mmHg. Hypotension was maintained until the animals could no longer maintain a MAP of 35 mmHg unless some fluid in the form of Ringer lactate solution was given to maintain until the animals could no longer maintain a MAP of 35 mmHg. After the MBO, MAP was maintained between 35 and 40 mmHg by giving small volumes of Ringer lactate until 40% of the MBO volume was returned in the form of Ringer lactate solution (∼90 min from the onset of bleeding). The rats were then resuscitated with four times the volume of MBO with Ringer lactate over 60 min, irrespective of whether they received flutamide or vehicle at the beginning of resuscitation. Thus there was no difference in the MBO or blood volume withdrawn for attaining MBO among the different groups. After resuscitation, the catheters were removed and the wounds were closed. Sham-operated animals underwent the surgical procedures, except that neither hemorrhage nor resuscitation was carried out. The animals were killed 2 h after the end of resuscitation or sham operation. In the treatment group, flutamide (25 mg/kg body wt; Sigma, St. Louis, MO) was administered subcutaneously at the beginning of the resuscitation. In another group of rats, ICI-182,780 (3 mg/kg body wt; Sigma) was given intraperitoneally at the beginning of the resuscitation or without flutamidetreatment. In the combined treatment group, flutamide was administered 10 min after the administration of ICI-182,780. In the vehicle-treated group, the rats received the same volume of vehicle (1 ml/kg body wt, corn oil, 10% DMSO, or 1:2-propanediol; Sigma) subcutaneously.

Determination of cardiac function. At 2 h after trauma-hemorrhage or sham operation, the animals were anesthetized with pentobarbital sodium (30–50 mg/kg ip). Cardiac output (CO) was determined with the use of the indocyanine green (ICG) dilution technique (5). A 2.4-Fr fiber-optic catheter (Hospex Fiberoptics, Chestnut Hill, MA) was inserted to the level of the aortic arch for continuous measurement of ICG concentration with the use of an in vivo hemoreflectometer (Schwarzer-Picker International, Munich, Germany). A silicone rubber catheter was inserted into the right atrium. ICG (50 μl, 1 mg/ml) was injected via the right jugular vein catheter. The concentration of ICG was recorded by a computer-assisted data acquisition program (Asystant+, Asyst Software, Rochester, NY). After the measurement of CO, the right carotid artery was cannulated with PE-50 PMMA tube (Clay-Adams, Inc., Hackettstown, NJ) advanced into the left ventricle and connected to a heart performance analyzer (DigiMed) to monitor and record positive and negative first derivatives of pressure (+dP/dtmax and −dP/dtmax), CO, stroke volume, and total peripheral resistance were calculated according to standard equations.

Determination of cardiac sex hormone levels. Heart tissues were homogenized in phosphate buffer, and tissue lysates were then centrifuged at 10,000 g for 10 min. After centrifugation, the resulting supernatants were assayed for sex hormone contents. The estradiol, testosterone, (Cayman Chemical, Ann Arbor, MI), and DHT (Alphaco Diagnostics, Windham, NH) were determined by enzyme immunoassay kits.

Aromatase activity. The aromatase activity was performed as described previously (23). Briefly, heart tissues were homogenized in 3 volume 50 mM Tris-maleate buffer, pH 7.4, containing (in mM) 1 β-mercaptoethanol, 40 nicinamide, and 250 sucrose. [1H]Androstenedione was used as substrate. The assay mixture consisted of 10 mM NADPH, 50 mM glucose-6-phosphate, and 62.5 U glucose-6-phosphate dehydrogenase. After 1 h incubation at 37°C, the reaction was terminated by the addition of 2 volume chloroform and then 1 ml 10% activated charcoal with 1% dextran T70 was added for H2O release. After centrifugation at 10,000 g for 10 min, the radioactivity in 500 μl of supernatant was measured in a scintillation counter (Wallac, Gaithersburg, MD).

Western blot analysis. The heart tissues were homogenized in lysis buffer consisting of 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.5 mM phenylmethylsulfonylfluoride, 1 mM sodium vanadate, 1% Triton X-100, 0.5% Nonidet P-40, and 1 μg/ml of aprotinin. Tissue lysates were centrifuged at 17,000g for 10 min. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). Protein aliquots were mixed with 4× lysis buffer and run on a 4–12% SDS-polyacrylamide gels, and transferred electrophoretically onto nitrocellulose paper. The membranes were immunoblotted with Sex-2reductase II (Santa Cruz Biotechnology, Santa Cruz, CA), ER-α, ER-β, AR (Upstate, Lake Placid, NY), and GAPDH (Abcam, Cambridge, MA) antibodies. This was followed by the addition of horse-radish peroxidase-conjugated secondary antibody. After the final wash, membranes were probed with the use of enhanced chemiluminescence (Amersham, Piscataway, NJ) and autoradiographed. GAPDH was used as a loading control.

Isolation of mitochondria. After the rats were killed, the hearts were removed, minced, and then homogenized gently in 0.25 mol/l sucrose, 0.5 mmol/l EGTA, and 3 mmol/l HEPES, pH 7.2, at the ratio of 10 mg/g of heart with a motor-driven grinder. The suspension was centrifuged at 750g for 10 min. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). Protein aliquots were mixed with 4× lysis buffer and run on a 4–12% SDS-polyacrylamide gels, and transferred electrophoretically onto nitrocellulose paper. The membranes were immunoblotted with Sex-2reductase II (Santa Cruz Biotechnology, Santa Cruz, CA), ER-α, ER-β, AR (Upstate, Lake Placid, NY), and GAPDH (Abcam, Cambridge, MA) antibodies. This was followed by the addition of horse-radish peroxidase-conjugated secondary antibody. After the final wash, membranes were probed with the use of enhanced chemiluminescence (Amersham, Piscataway, NJ) and autoradiographed. GAPDH was used as a loading control.

COX assay. The COX activity of cardiac mitochondria was analyzed by an assay kit (Sigma) according to the manufacturer’s instructions. Briefly, isolated mitochondria were homogenized in assay buffer (10 mM Tris-HCl, pH 7.0, and 120 mM KCl). Approximately 500 μg of mitochondrial protein were added to 1.1 ml of reaction solution, which contained 50 μl of 0.22 mM fully reduced ferrocyanochrome c, 10 mM Tris-HCl (pH 7.0), and 120 mM KCl. The decrease of absorbance at 550 nm was recorded during a 1-min reaction time.

Determination of mitochondrial ATP levels. ATP content was measured by the ATP BIoluminescence Assay kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. Briefly, the mitochondrial pellet was suspended in lysis reagent supplied in the kit. The suspensions were pipetted and vortexed, and the protein concentrations were measured (Bio-Rad Laboratories). The samples were kept on ice until measurements were performed. For determination of ATP, 100 μl of luciferase reagent were added to the standards or 1 mg of mitochondrial proteins, and the measurement was performed with a luminometer AutoLumat LB983 (Berthold, Wildbad, Germany).

Quantitative real-time PCR. Total RNA was prepared by TriReagent (Life Technologies, Grand Island, NY), and 2 μg of total RNA were then reversed to cDNA by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Real-time PCR was performed with the use of the TaqMan™ method on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The sequences of primers and probes used in this study were designed with the ABI Prism Primer Express Program (Applied Biosystems), and they are as follows: 1) for COX I, 5′-agtatgeccat-
catagctgct-3’ and 5’-gcttttgctcatgtgcattaggg-3’, and probe 5’-gccagctcttattcttgagt-3’, and probe 5’-ccagctcttgcatcc-3’, and probe 5’-ccagctcttgcatcc-3’, and probe 5’-ccagctcttgcatcc-3’, and probe 5’-ccagctcttgcatcc-3’, The reaction mixture for TaqMan assay contained 5 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.1 μmol/l COX I and COX III, 0.2 μmol/l COX II primers, 0.25 μmol/l COX I, COX II, and COX III TaqMan probes, and 40 ng cDNA. All samples were tested in triplicate, and average values were used for quantification. 18S rRNA was used as an endogenous control. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye from the TaqMan probe. Analysis was performed with SDS version 2.2 software (Applied Biosystems) according to the manufacturer’s instructions. The comparative cycle threshold (CT) method (ΔΔCT) was used for quantification of gene expression.

Statistical analysis. All data are presented as means ± SE. One-way ANOVA and Tukey’s test were employed for the comparison among groups, and differences were considered significant at P < 0.05.

RESULTS

Cardiac function. As shown in Table 1, CO, stroke volume, +dP/dtmax, −dP/dtmax, total peripheral resistance, and MAP were significantly decreased (P < 0.05) after trauma-hemorrhage in vehicle-treated rats compared with sham-operated animals. Flutamide administration after trauma-hemorrhage restored all of the above parameters except MAP to levels comparable to those of the sham-operated group. Administration of the ER antagonist ICI-182,780 prevented the flutamide-mediated restoration of cardiac function after trauma-hemorrhage. No significant difference was observed in all cardiac parameters between nontreated sham and sham-operated animals treated with flutamide and ICI-182,780 or ICI-182,780 alone. This is in accordance with our previous studies (8, 35), which also showed that there was no significant difference in cardiac function between nontreated sham and sham-operated animals treated with ICI-182,780 or flutamide.

Alteration in cardiac sex steroids levels. There is no significant difference in cardiac testosterone (Fig. 1A) and estrogen (Fig. 1B) levels in the sham-operated and trauma-hemorrhage rats treated with vehicle or ICI-182,780. Flutamide administration after trauma-hemorrhage, however, significantly increased testosterone and estrogen levels compared with vehicle-treated trauma-hemorrhage group. The flutamide-induced increase in estrogen levels was, however, not observed in the trauma-hemorrhage group treated with flutamide and ICI-182,780. The flutamide-induced increase in testosterone levels remains elevated by coadministration of ICI-182,780 (Fig. 1; P < 0.05). Cardiac DHT levels were unaffected by trauma-hemorrhage or treatment of trauma-hemorrhage with either flutamide and ICI-182,780 or ICI-182,780 alone (ICI). *P < 0.05 vs. sham and T-H-Veh; †P < 0.05 vs. T-H-FL.

Cardiac steroidogenic enzyme activity and expression. The cardiac aromatase activity was not significantly different between the sham-operated and vehicle-treated trauma-hemorrhage groups. Levels of aromatase activity were lower in the ICI-182,780-treated groups compared with the vehicle-treated groups. Flutamide administration increased aromatase activity in all groups, and the activity was highest in the ICI-182,780-treated group. These results suggest that flutamide administration after trauma-hemorrhage may increase aromatase activity, which is consistent with the increase in estrogen levels observed in the experimental groups.

Table 1. Effect of treatment of rats with flutamide alone or in combination with ICI-182,780 on cardiac function after trauma-hemorrhage

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>S+FL+ICI</th>
<th>Veh</th>
<th>FL</th>
<th>FL+ICI</th>
</tr>
</thead>
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<tr>
<td>CO, ml/min⁻¹·100 g body wt⁻¹</td>
<td>41.5±1.3</td>
<td>43.0±1.5</td>
<td>26.1±1.8*</td>
<td>37.5±1.2</td>
<td>19.7±0.7†</td>
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<td>SV, μl/beat⁻¹·100 g body wt⁻¹</td>
<td>94.1±1.7</td>
<td>93.7±2.7</td>
<td>73.5±0.6*</td>
<td>105.2±3.4</td>
<td>68.0±4.8†</td>
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<td>+dP/dtmax, mmHg/s</td>
<td>15,290±1,452</td>
<td>15,885±1,270</td>
<td>8,162±821*</td>
<td>12,532±847</td>
<td>4,521±972†</td>
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<tr>
<td>−dP/dtmax, mmHg/s</td>
<td>8,944±1,005</td>
<td>8,757±322</td>
<td>4,411±371*</td>
<td>7,089±650</td>
<td>2,568±333*</td>
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<td>MAP, mmHg</td>
<td>120±3</td>
<td>121±2</td>
<td>74±6*</td>
<td>92±4*</td>
<td>65±15†</td>
</tr>
<tr>
<td>TPR, mmHg·min⁻¹·100 g body wt</td>
<td>2.55±0.13</td>
<td>2.56±0.06</td>
<td>3.17±0.18*</td>
<td>3.29±0.37</td>
<td>3.32±0.16†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals/group. S, sham operation; T-H, trauma-hemorrhage; CO, cardiac output; SV, stroke volume; +dP/dt and −dP/dt, positive and negative first derivatives of pressure; MAP, mean arterial pressure; TPR, total peripheral resistance. T-H group treated with vehicle (Veh), flutamide (FL), FL and ICI-182,780 (FL+ICI), or ICI-182,780 alone (ICI). *P < 0.05 vs. sham; †P < 0.05 vs. T-H-FL.
Cardiac sex steroid receptor expression. The protein levels of ER-α and ER-β decreased significantly \((P < 0.05)\) after trauma-hemorrhage compared with the sham-operated group (Fig. 3, A and B). Flutamide administration after trauma-hemorrhage normalized both ER-α and ER-β protein levels to sham values. However, coadministration of ICI-182,780 prevented the flutamide restoration of ER-α and ER-β in trauma-hemorrhage rats. Similarly, administration of ICI-182,780 alone or with flutamide after trauma-hemorrhage had no salutary effect on ER-α or ER-β protein levels. There was no significant difference in cardiac AR protein expression between the trauma-hemorrhage and sham-operated groups (Fig. 3C), and flutamide treatment or administration of flutamide and ICI-182,780 or ICI-182,780 alone also had no effect on AR expression.

Cardiac PGC-1 mRNA expression. The mRNA expression of PGC-1 decreased significantly \((P < 0.05)\) after trauma-hemorrhage compared with the sham-operated group (Fig. 4). Flutamide administration after trauma-hemorrhage normalized PGC-1 mRNA expression to sham values. However, coadministration of ICI-182,780 prevented the flutamide restoration of PGC-1 in trauma-hemorrhage rats. Furthermore, administration of ICI-182,780 per se after trauma-hemorrhage had no salutary effect on PGC-1 mRNA.

Cardiac mtDNA-encoded COX I, COX II, and COX III gene expressions, mitochondrial COX activity, and ATP levels. To evaluate the PGC-1 downstream effects, we measured mtDNA-encoded COX I, COX II, and COX III gene expressions, mitochondrial COX activity, and mitochondrial ATP levels. The results showed that mtDNA-encoded COX I, COX II, COX III gene expressions (Fig. 5, A–C), and mitochondrial COX activity (Fig. 5D), mitochondrial ATP levels (Fig. 6) decreased significantly \((P < 0.05)\) in the vehicle-treated trauma-hemorrhage group compared with the sham-operated group. Flutamide administration after trauma-hemorrhage increased COX I gene expression and COX activity, and ATP levels were similar to those observed in the sham-operated animals. However, administration of ICI-182,780 abolished the flutamide normalization of COX I gene expression, COX activity, and ATP levels in trauma-hemorrhage rats. Likewise, admin-

![Fig. 2. Cardiac steroidogenetic enzyme aromatase activity (A) and 5α-reductase II protein expression (B) in S and T-H rats treated with FL alone or in combination with ICI. Values are means ± SE of 6 animals in each group. Results were compared by one-way ANOVA and Tukey’s test. T-H group treated with Veh, FL, FL+ICI, or ICI. *P < 0.05 vs. sham and T-H-Veh; #P < 0.05 vs. T-H-FL.](http://ajpheart.physiology.org/ by 10.220.33.4 on October 23, 2017)
The findings of this study reveal that cardiac function; cardiac ER-α and ER-β protein levels; PGC-1; mtDNA-encoded COX I, COX II, and COX III mRNA expressions; mitochondrial COX activity; and mitochondrial ATP levels were markedly decreased at 2 h after trauma-hemorrhage. Administration of flutamide after trauma-hemorrhage normalized cardiac function and induced the increases in cardiac testosterone, estrogen levels, and aromatase activity. This was accompanied by increased ER-α and ER-β, PGC-1, mtDNA-encoded COX I gene expressions, mitochondrial COX activity, and ATP levels. In contrast, cardiac DHT, 5α-reductase II, AR protein levels, and mtDNA-encoded genes COX II and COX III were unaffected by flutamide or ICI-182,780 administration. However, administration of ER antagonist ICI-182,780 along with flutamide after trauma-hemorrhage prevented flutamide-mediated restoration of cardiac function, the increase in aromatase activity and estrogen levels, ER-α, ER-β protein levels, PGC-1, mtDNA-encoded COX I gene expressions, mitochondrial COX activity, and ATP levels. These findings, therefore, suggest that flutamide increases estrogen levels in the heart by increasing the conversion of testosterone to estrogen, thereby increasing ER-α and ER-β, which in turn upregulates PGC-1 and restores mitochondrial ATP production, thus preventing cardiac depression after trauma-hemorrhage. Thus the novel findings of this study are that our results clearly indicate that the beneficial effects of flutamide after trauma-hemorrhage are mediated via an estrogen-dependent pathway through upregulation of PGC-1.

Although our previous study (35) has shown that administration of flutamide improves cardiac function and increases ER expression in cardiomyocytes after trauma-hemorrhage, it remains unknown whether the salutary effects of flutamide are mediated via ER. In this study, we further confirmed that the flutamide-mediated restoration of cardiac function was not observed if flutamide was administered with ICI-182,780, an ER antagonist. This finding clearly indicated that the salutary effects of flutamide on cardiac function after trauma-hemorrhage are due to upregulation of ER expression. Studies have reported that male rats treated with flutamide had increased plasma testosterone and estrogen concentration (17, 19, 33). It is also known that testosterone is converted to estrogen and DHT by aromatase and 5α-reductase, respectively, which subsequently interacts with ER or AR, respectively (1, 25, 29). In the present study, we found that flutamide administration after trauma-hemorrhage increased testosterone and estrogen levels as well as aromatase activity in the heart; however, flutamide-increased aromatase activity and estrogen levels were abolished when flutamide was administered with ICI-182,780. Although flutamide administration after trauma-hemorrhage increased cardiac testosterone levels, there were no effects on cardiac DHT and 5α-reductase II protein levels. These findings, therefore, suggest that administration with flutamide after trauma-hemorrhage increases estrogen levels in the heart by increasing the conversion of testosterone to estrogen. This conclusion is supported by the fact that flutamide-treated animals had increased cardiac aromatase protein, and it is well
known that the enzyme aromatase converts testosterone to estradiol (24).

It is well known that estrogen mediates its effects via its receptors ER-α and ER-β (15, 28). In the present study, we found that cardiac ER protein levels significantly decreased after trauma-hemorrhage; however, administration of flutamide after trauma-hemorrhage normalized cardiac ER-α and ER-β levels. These findings corroborate our previous results, which showed that treatment of flutamide after trauma-hemorrhage upregulated ER on cardiomyocytes and splenic T lymphocytes (24, 35). However, the present study also demonstrated that the flutamide-mediated upregulation of cardiac ER-α and ER-β was abolished by administration of ICI-182,780 along with flutamide after trauma-hemorrhage. These changes in ER expressions were accompanied by the alterations in aromatase activity and estrogen levels in the heart, suggesting that flutamide-mediated upregulation of cardiac aromatase activity contributes to increased estrogen levels in the heart that in turn upregulate cardiac ER-α and ER-β after trauma-hemorrhage. Nonetheless, the precise mechanism by which flutamide upregulates ER and restores cardiac function after trauma-hemorrhage remains unclear. In view of this, we further explored the possibility that the upregulation of ER may influence the expression of PGC-1 and its downstream effects.

The nuclear coactivator PGC-1, which is highly expressed in the heart and skeletal muscle, has been characterized as a key regulator of mtDNA-encoded gene expressions, such as COX I, COX II, COX III, and an important regulator of ATP production in the heart (12, 20, 26, 27). Although our recent study (6) has also found that flutamide increased PGC-1 protein levels through ER after trauma-hemorrhage, the precise mechanism by which that occurs still remains unclear. In this study, we further demonstrated that flutamide-increased cardiac PGC-1, COX I mRNA expression, mitochondrial COX activity, and ATP levels were prevented by coadministration with ICI-182,780 after trauma-hemorrhage, suggesting that the upregulation of PGC-1 in flutamide-treated trauma-hemorrhage rats restored mitochondrial ATP production. However, because COX II and COX III were not affected by flutamide administration after trauma-hemorrhage, it indicates that flutamide increased COX activity due to COX I rather than COX II and COX III. These findings reveal that flutamide-mediated restoration of cardiac function is via an estrogen-dependent pathway through which PGC-1 upregulated mtDNA-encoded gene COX I and COX activity and restored ATP production after trauma-hemorrhage.
It could be argued that the present study utilized measurement at a single time point, i.e., at 2 h after administration of flutamide, and thus it remains unclear whether the salutary effects are sustained for longer periods of time, i.e., 24 h after treatment. In this regard, our previous studies (3, 8, 9, 16, 31, 34) have shown that if the improvement in organ function by any pharmacological agent is evident early after treatment that those salutary effects are sustained for prolonged intervals and that they also improve the survival of animals. Thus, although a time point other than 2 h was not examined in this study, on the basis of our previous studies, it would appear that the salutary effects of flutamide would be evident even if one measured those effects at another time point after trauma-hemorrhage and resuscitation. It could also be argued that plasma sex steroid levels should have been measured to determine whether flutamide had any effect on the sex steroids. Measurement of sex steroids in circulation was indeed carried out, and the plasma level of the sex steroids also followed the same trend as that seen in the cardiac tissue. However, because cardiac tissue sex steroid levels are more meaningful, we have not included the plasma sex steroid levels in the results.

The studies of Pelzer et al. (18) using ER-β knockout mice indicate that such mice are more prone to heart failure than are wild-type mice. This suggests that ER-β plays a major role in preventing heart failure under normal conditions (18). In view of these findings, it can also be suggested that ER-β also plays a major role after trauma-hemorrhage. However, it remains to be determined whether ER-α or ER-β plays a major role in the heart after trauma-hemorrhage. Because the specific ER-α and ER-β agonists are available, we hope to determine in the future whether ER-α or ER-β agonists per se can improve cardiac function after trauma-hemorrhage.

Our previous results (31) have shown that flutamide administration after trauma-hemorrhage improved the depressed immune function and that it also decreased the proinflammatory cytokine release. Moreover, our studies (3) have shown that flutamide induces relaxation in small and large blood vessels. The present study shows that flutamide also improves cardiac function. However, because salutary effects of flutamide were also observed on the immune functions and vascular reactivity, it is possible that improvement in the function of other organs in addition to the salutary effects on cardiac function also plays a role in the overall improvement of the animal after trauma-hemorrhage. Nonetheless, because blood pressure was not restored to normal with flutamide administration, it is possible that it takes a longer period of time to restore blood pressure than the 2-h interval used in this study.

In summary, our results indicate that the administration of flutamide after trauma-hemorrhage restores depressed cardiac function and induces increases in cardiac testosterone, estrogen levels, aromatase activity, ER-α, ER-β, PGC-1, COX I, mitochondrial COX activity, and ATP production in the heart. In contrast, flutamide did not affect cardiac DHT, 5α-reductase II, and AR protein levels after trauma-hemorrhage. Furthermore, administration of ICI-182,780 with flutamide prevented the flutamide-mediated restoration of cardiac function and the increases in cardiac testosterone, estrogen levels, aromatase activity, ER-α, ER-β, PGC-1, COX I, mitochondrial COX activity, and ATP production. These findings collectively suggest that flutamide prevents cardiac dysfunction after trauma-hemorrhage via an estrogen-dependent pathway through up-regulation of PGC-1.

GRANTS

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