Estrogen Is Renoprotective *via* a Nonreceptor-dependent Mechanism after Cardiac Arrest *In Vivo*

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ABSTRACT

Background: Severe ischemia induces renal injury less frequently in women than men. In this study, cardiac arrest and cardiopulmonary resuscitation were used to assess whether estradiol is renoprotective *via* an estrogen receptor (ER)-dependent mechanism.

Materials and Methods: Male and female C57BL/6 and ER gene-deleted mice underwent 10 min of cardiac arrest followed by cardiopulmonary resuscitation. Serum chemistries and renal stereology were measured 24 h after arrest.

Results: Estrogen did not affect mean arterial pressure, regional renal cortical blood flow, and arterial blood gases. Hence, female kidneys were protected (mean ± SEM: blood urea nitrogen, 65 ± 21 *vs.* 149 ± 27 mg/dl, $P = 0.04$; creatinine, 0.14 ± 0.05 *vs.* 0.73 ± 0.16 mg/dl, $P = 0.01$; volume of necrotic tubules, 7 ± 1% *vs.* 10 ± 0%, $P = 0.04$). Estrogen also reduced renal injury. In intact females (n = 5), ovariectomized/vehicle-treated (n = 8), and ovariectomized/estrogen-treated (n = 8) animals, blood urea nitrogen was 65 ± 21, 166 ± 28, and 50 ± 14 mg/dl ($P = 0.002$); creatinine was 0.14 ± 0.05, 0.74 ± 0.26, and 0.23 ± 0.27 mg/dl ($P = 0.014$); necrotic tubules were 2.5 ± 0.25%, 12.0 ± 1.9%, and 5.0 ± 1.6% ($P = 0.004$), respectively. In ER-α and ER-β gene-deleted mice and controls estradiol-reduced functional injury (blood urea nitrogen: estradiol 117 ± 71, vehicle 167 ± 56, $P = 0.007$; creatinine: estradiol 0.5 ± 0.5, vehicle 1.0 ± 0.4, $P = 0.013$), but the effect of estradiol was not different between ER-α or ER-β gene-deleted mice. Adding ICI 182,780 to estradiol did not alter injury.

Conclusions: In women, kidneys were protected from cardiac arrest through estrogen. Estradiol-mediated renoprotection was not affected by ER deletion or blockade. Estradiol is renoprotective after cardiac arrest. The results indicate that estradiol renoprotection is ER-α and ER-β independent.

What We Already Know about This Topic

❖ Acute kidney injury in the critically ill is less common in women

❖ Estrogen is protective to regional renal ischemia, but whether this applies to global ischemia from cardiac arrest is unknown

What This Article Tells Us That Is New

❖ Renal injury after cardiac arrest and resuscitation was reduced in female mice and was dependent on estrogen

❖ Estrogen receptor blockade did not affect this protection, indicating an effect by estrogen outside signaling by estrogen receptors α and β

A CUTE kidney injury (AKI) is a common complication of critical illness[1,2] and imposes a mortality of 50–70%.[3–5] Notably, AKI is less common[6] and less severe[7] in women. Although multiple strategies and protective agents have been used in an attempt to reduce the incidence of AKI in critically ill patients, none have been successful. Thus, there is compelling rationale to investigate the mechanism responsible for the well-known sex difference in renal outcomes after ischemia.

Sex steroids, and in particular, estradiol, have been shown to mediate ischemia–reperfusion injury in multiple organs and have been a target for investigation of the sex difference in renal ischemia–reperfusion injury. Testosterone exacerbates renal ischemia–reperfusion injury in focal (renal pedicle occlusion) ischemia.[8] A sex difference and a salutory effect of estradiol administration have been demonstrated in the pedicle occlusion model,[9] but it is not known whether these effects are present in whole body ischemia. Findings from global and focal ischemia models have been divergent in both cerebral and cardiac outcomes.[10,11] If this also true for outcomes in kidney, then generalizing mechanistic findings or therapeutic trials from focal to global renal ischemia (*e.g.*, from the experimental model of renal pedicle occlusion to the clinical situation of hypotension with global no or low flow) could have unanticipated results.

Estrogen induces both rapid nongenomic, nonreceptor-mediated effects and receptor-mediated transcriptional ef-

fects.[12] Because even the chronic receptor-mediated effects occur over hours to days, estrogen's amelioration of renal ischemia may be receptor mediated or nonreceptor mediated. Two estrogen receptor (ER) subtypes, ER-α and ER-β, have been identified. If a specific receptor subtype is responsible for a protective effect, then using selective ER modulators to specifically target the protective receptor may have important therapeutic implications. These agents have less dangerous side effect profiles than estrogen and might be more safely administered in the perioperative period.

Accordingly, we tested the hypothesis that estrogen is renoprotective in global ischemia *via* an ER-mediated pathway. Using normothermic cardiac arrest and cardiopulmonary resuscitation (CA/CPR) in the mouse, an established model of whole body ischemia–reperfusion injury,[13] we assessed sex difference and interrogated for receptor dimorphism by testing animals with ER-α or ER-β gene deletions. We also evaluated renal functional impairment and histopathologic outcomes.

Materials and Methods

This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research, and all animal protocols were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee, Portland, Oregon.

Animals and Experimental Groups

Male and female C57BL/6 mice were obtained from Charles River Laboratories in Boston, MA. We used females from ER-α and ER-β knockout (ErαKO and ERβKO) and wild-type (WT) mouse strains bred in our laboratory colonies, as previously described.[14–17] Both strains were bred to confluence in our laboratory colonies from back crosses with the parent C57BL/6-J mouse strain.

Protocol 1: Effect of Estradiol on Mean Arterial Pressure during CA/CPR. Ovariectomized female C57BL/6 mice were treated with either 17β-estradiol or vehicle and subjected to CA/CPR (n = 5/group). Mean arterial pressure was measured using a polyethylene-10 catheter placed in the femoral artery and was recorded 5 min before CA, immediately before CA, every minute during the 10-min period of CA, and every 5 min for 20 min after return of spontaneous circulation.

Protocol 2: Effect of Estradiol on Regional Renal Cortical Blood Flow during CA/CPR. Ovariectomized female C57BL/6 mice were treated with either 17β-estradiol or vehicle and subjected to CA/CPR (n = 5 or 6/group). A laser Doppler flow probe was placed perpendicular to the surface of the right kidney *via* a flank incision. The probe tip was immersed in isotonic sodium chloride solution and optimally positioned for maximal signal. Laser Doppler flow was measured and recorded 5 min before CA, immediately before CA, every minute during CA, and every 5 min after CA for 20 min after return of spontaneous circulation. The averages of the measurements taken 5 min before and immediately before CA served as baseline flow, and all measurements were re-

corded as a percent of baseline flow to minimize variation between animals.

Protocol 3: Effect of Estradiol on Blood Chemistry before and after CA/CPR. To ensure that phlebotomy-induced anemia did not affect other experimental groups, a separate cohort of ovariectomized female C57BL/6 mice were treated with either 17β-estradiol or vehicle and subjected to CA/CPR (n = 5/group). A femoral arterial catheter was placed before CA 5 min after return of spontaneous circulation, and again 10 min later, 100 μl of arterial blood was aspirated and analyzed using an automated blood gas analyzer (Radiometer 1200, Bayer Healthcare, Norwood, MA). Because anemia induced by the baseline phlebotomy could conceivably affect survival from CA/CPR or alter post-CA/CPR blood gas analysis, a separate cohort (n = 5/group) underwent general anesthesia and placement of arterial catheters for baseline arterial blood gas analysis.

Protocol 4: Effect of CA/CPR on Urine Neutrophil Gelatinase-associated Lipocalin. Ovariectomized female C57BL/6 mice (n = 9/group) were treated with vehicle and subjected to CA/CPR. Immediately before CA, urine was expressed by gentle low abdominal pressure and then aspirated into a syringe. At 24 h after CA/CPR, general anesthesia was induced with 1.5% isoflurane and 1 ml of isotonic sodium chloride solution administered subcutaneously. After 30 min, urine was again expressed and aspirated, and euthanasia, transcardial perfusion, and kidney harvest were performed after transcardial perfusion.

Protocol 5: Sex Difference. Gonadally intact male and female C57BL/6 mice (n = 10/group) were subjected to CA/CPR. At 24 h after CA/CPR, deep anesthesia was induced using 1.5% isoflurane. A blood sample was then collected for blood urea nitrogen (BUN) and creatinine, and kidney harvest was performed after transcardial perfusion.

Protocol 6: Effect of Estradiol on Renal Injury after CA/CPR. Three groups were assessed. Gonadally intact, ovariectomized vehicle treated and ovariectomized 17β-estradiol treated C57BL/6 mice (n = 5–8/group) were subjected to CA/CPR. At 24 h after CA/CPR, deep anesthesia was induced using 1.5% isoflurane. A blood sample was then collected for BUN and creatinine, and kidney harvest was performed after transcardial perfusion.

Protocol 7a: Role of Classic ERs in Estradiol-mediated Renoprotection Assessed Using ERα and ERβ Gene-deleted Mice. A total of eight groups were assessed. Ovariectomized female ER-α and ER-β gene-deleted mice and their respective WT littermate controls (n = 4–14) were treated with either 17β-estradiol or vehicle and subjected to CA/CPR. At 24 h after CA/CPR, deep anesthesia was induced using 1.5% isoflurane. A blood sample was then collected for BUN and creatinine, and kidney harvest was performed after transcardial perfusion.

Protocol 7b: Role of Classic ERs in Estradiol-mediated Renoprotection Assessed Using ICI 182,780, a Classic ER Antagonist. Ovariectomized female C57BL/6 mice were treated with either 17β-estradiol or vehicle and ICI 182,780

and subjected to CA/CPR. At 24 h after CA/CPR, deep anesthesia was induced using 1.5% isoflurane. A blood sample was then collected for BUN and creatinine, and kidney harvest was performed after transcardial perfusion.

In Vivo *Whole Body Ischemia–Reperfusion Injury*
We conducted CA/CPR as previously described.[18–20] Mice were removed from their home cages in random order with respect to their strain or treatment. Anesthesia was induced with 4% isoflurane and was subsequently maintained with 1–2% isoflurane in air/oxygen mixture. Mice were weighed, positioned on the operating table, and mechanically ventilated after tracheal intubation with a 22-gauge catheter. Body temperature was monitored with a rectal probe and maintained at $37.0° \pm 0.5°C$ with a heating lamp and warm pad. A catheter was inserted into the right jugular vein. In some groups, urine was expressed or a femoral arterial catheter or a laser Doppler flow probe was placed at this time. The electrocardiogram was monitored with subdermal electrodes, and CA was induced with 40 μl iced 0.5 M KCl intravenously and confirmed by electrocardiography. Ventilation was stopped and the endotracheal tube was disconnected from the ventilator. After 9.5 min of normothermic CA with no ventilation, the endotracheal tube was reconnected to the ventilator, and hyperventilation at 120% of prearrest rate was initiated using 100% O_2. At 10 min, chest compressions were initiated at a rate of 300/min, and epinephrine (8–15 μg in 0.5–1 ml isotonic sodium chloride solution) was administered intravenously in divided doses. CPR was discontinued on return of spontaneous circulation as observed on the electrocardiograph, or after 4 min of CPR without return of spontaneous circulation. Electrocardiographic evidence of return of spontaneous circulation is confirmed after cessation of CPR by observation of cardiac contractions that are visible on the chest wall. Animals were extubated when spontaneous respiratory rate was greater than 60/min, usually 12–18 min after return of spontaneous circulation. The jugular catheter was removed, hemostasis obtained, and animals returned to cages. The recovery cage was placed on a warming mat set at 37°C to maintain normothermia in the postarrest period. Surviving animals were deeply anesthetized 24 h after CA, and tissue was fixed *via* transcardial perfusion for subsequent renal histologic analysis.

Transcardial Perfusion and Kidney Harvest
At 24 h after CA/CPR, general anesthesia was induced with isoflurane and transcardial perfusion was performed using 4% paraformaldehyde in saline. Kidney harvest was then performed immediately *via* postmortem midline laparotomy.

Histologic Preparation and Stereological Analysis
After fixation in 4% paraformaldehyde in saline, each kidney was sectioned in the sagittal plane into 6-μm sections at four equidistant locations along the long axis of the kidney. These were then stained with Flouro-Jade B (Histo-Chem, Jefferson, AK) to clearly delineate necrotic *versus* nonnecrotic cells.

An observer blinded to sex, strain, and treatment assessed the volume fraction of necrotic tubular epithelium according to the Cavalieri principle of unbiased stereology.[21,22] Computer software (Visiopharm Integrator System, Visiopharm, Hørsholm, Denmark) was used with an X-Y-Z-controlled microscope stage and video camera (Leica Microsystems GMbH, Wetzlar, Germany). The software automatically and randomly positioned two superimposed grids of points (one of low resolution and the other of high resolution) over each of the displayed video images from the microscope. There are 16 high-resolution points per low-resolution point. $\sum P$(kidney) (the reference space) is determined by counting the number of low-resolution points that intersect the kidney and multiplying by 16. $\sum P$(necrotic tubular epithelium) is determined by counting the number of high-resolution points that intersect necrotic epithelium. The estimated volume fraction of necrotic tubular epithelium is the ratio of these two quantities according to the following equation:

$$V_{\text{necrotic tubular epithelium}} = \frac{\sum P(\text{necrotic tubular epithelium})}{\sum P(\text{kidney})}$$

Surgical and Pharmacologic Hormonal Manipulation
In protocols in which estradiol was used, but not ICI 182,780, ovariectomy was performed, and estradiol or sesame oil (vehicle) pellets were simultaneously implanted 7 days before CA/CPR. The estradiol subcutaneous silastic implants contained 35 μl of 180 μg/ml estradiol in sesame oil (6.3 μg total dose). This estradiol dose has been used previously by our group and yields physiologic levels of plasma estradiol comparable with cycling female mice.[23]

Because we were unable to find precedent in the scientific literature for delivering ICI 182,780 *via* silastic implant, we used osmotic pumps (ALZET 1007D, Durect Corp, Cupertino CA) in Protocol 7b. Pumps were filled with 6.3 μg 17β-estradiol in 50% dimethylsulfoxide/0.9% saline solution or with 6.3 μg 17β-estradiol and 700 μg ICI 182,780. This dose of ICI 182,780 was chosen based on previous work, showing that it antagonizes the effects of physiologic concentrations of estradiol.[24,25] Ovariectomy was performed and the pumps were implanted *via* the same incision in a subcutaneous pocket along the left flank. Seven days after implant, CA/CPR was performed, and 24 h later, transcardial perfusion and sample acquisition were carried out.

BUN and Serum Creatinine Assay
Blood was drawn from the apex of the left ventricle at the time of euthanasia and placed in lithium heparin tubes and then analyzed for urea nitrogen and creatinine, using an enzyme-coupled point-of-care analyzer (Abaxis Medical Diagnostics, Union City, CA). The creatinine amidohydrolase catalyzed assay used by this device is more specific for creatinine than the commonly used Jaffe technique,[26–29] which is altered by chromogens present in mouse blood samples.[30]

Neutrophil Gelatinase-associated Lipocalin Western-blot Assay

Anti-mouse neutrophil gelatinase-associated lipocalin (NGAL) antibodies were purchased from R&D Systems (Minneapolis, MN). Urine samples (7.5 μl) were thawed on ice and boiled for 5 min at 90°C in 1× sample buffer (Invitrogen, Carlsbad, CA) before loading onto a 12% Nupage Bis-Tris gel (Invitrogen) and electrophoresed for 50 min at 200 V. Gels were then transferred to polyvinylidene fluoride membranes using 30 V for 2 h. The polyvinylidene fluoride membranes were then extensively washed in phosphate-buffered saline with Tween-20, blocked, and incubated with the primary antibody (diluted 1:500) overnight at 4°C. After washing, membranes were then incubated for 2 h at room temperature with ECL-Plex Cy3-conjugated secondary antibody (GE Life Sciences, Piscataway, NJ) diluted at 1:750. Immunoreactive bands were visualized using the Typhoon 9400 imager (GE Life Sciences) and analyzed using Image-Quant software (GE Life Sciences). Band densities were normalized to a single control sample run on every gel under identical conditions.

Data Analysis

Analysis was performed with Prism 5.0 software (GraphPad Software, LaJolla, CA). All data are shown as mean ± SEM. For all statistical tests, significance was inferred at $P < 0.05$. Physiologic data were analyzed using one-way analysis of variance with Tukey *post hoc* test for intergroup comparisons. Contingencies (mortality) were analyzed using Fisher exact test. NGAL and sex difference comparisons were made using Student t test with two-tailed P values. The comparison between intact females, ovariectomized, vehicle-treated females, and ovariectomized 17β-estradiol-treated females was performed using one-way analysis of variance with Tukey *post hoc* comparison test. Two-way analysis of variance was used for analysis of the four-group/two-treatment genetic ER-deletion protocol. Because no statistically significant difference was found on two-way analysis of variance, posttesting was not conducted on data from this protocol.

Results

Weight, epinephrine dose required, pre- and intraarrest temperature, and mortality were not different between groups (table 1). There was no difference in mean arterial pressure or regional renal cortical blood flow (RRCBF) between 17β-estradiol-treated and vehicle-treated animals (fig. 1). Arterial blood gas parameters were similar between 17β-estradiol-treated and vehicle-treated groups (table 2). The duration of CPR was different between the ERαKO vehicle- and 17β-estradiol-treated groups (48 ± 5 *vs.* 70 ± 14 s, $P = 0.045$). At the time of tissue harvest, 17β-estradiol levels were 20 ± 3 pg/ml in untreated ovariectomized animals and 70 ± 11 pg/ml in estradiol-treated ovariectomized animals ($P = 0.0006$).

Table 1. Cardiac Arrest Data

Group (N)	Weight, g	Epinephrine Dose, μg	CPR Duration, s
ERβKO-VEH (14)	20.3 ± 0.2	11.0 ± 0.5	70.1 ± 5.2
ERβKO-EST (10)	21.8 ± 0.7	10.4 ± 1.3	62.5 ± 9.3
ERβWT-VEH (6)	20.3 ± 0.4	9.5 ± 1.1	65.5 ± 9.8
ERβWT-EST (11)	20.8 ± 0.5	10.0 ± 1.8	60.5 ± 7.0
ERαKO-VEH (5)	20.6 ± 1.0	9.6 ± 0.4	48.2 ± 4.9*
ERαKO-EST (5)	21.0 ± 1.6	10.7 ± 0.7	69.8 ± 13.6
ERαWT-VEH (5)	20.4 ± 0.5	11.5 ± 0.8	66.6 ± 13.2
ERαWT-EST (4)	21.1 ± 1.0	8.8 ± 0.3	56.5 ± 9.0
NGAL (10)	22.4 ± 0.4	10.9 ± 0.4	51.7 ± 3.6
Intact female (5)	23.3 ± 1.2	10.4 ± 0.8	41.2 ± 9.1
OVX-VEH (8)	22.7 ± 0.5	9.9 ± 0.5	39.3 ± 5.7
OVX-EST (8)	23.0 ± 0.4	9.9 ± 0.5	42.5 ± 7.5
EST (12)	23.3 ± 0.3	7.1 ± 0.3	59.6 ± 8.1
EST-ICI (8)	22.6 ± 0.4	6.7 ± 0.4	55.4 ± 8.3
Intact male (10)	24.8 ± 0.5	11.3 ± 0.8	108.0 ± 11.9
Intact female (10)	21.4 ± 0.2	10.1 ± 0.7	84.2 ± 7.7

Parameters recorded during cardiac arrest. Data presented are mean ± SEM.

* $P < 0.05$ *vs.* ERαKO-EST.

CPR = cardiopulmonary resuscitation; ER = estrogen receptor; EST = estrogen; ICI = ICI 182,780; KO = knockout (gene deleted); NGAL = neutrophil gelatinase-associated lipocalin; OVX = ovariectomized; VEH = vehicle; WT = wild type.

Urine NGAL Is Massively Increased after CA

To further validate the model and show similarity to human AKI, we assessed pre- and postarrest urine NGAL, a clinically validated rapid biomarker in the perioperative setting.[31] Urine NGAL is increased after CA/CPR by nearly an order of magnitude (1.25 ± 0.41 *vs.* 9.49 ± 3.61, n = 9/group, $P = 0.04$; fig. 2, representative Western blot).
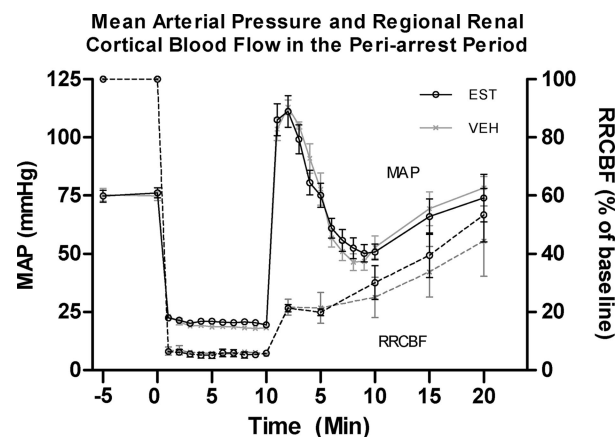

Fig. 1. Mean arterial pressure (MAP) and regional renal cortical blood flow (RRCBF) are not affected by administration of 17β-estradiol. MAP (*solid lines*, scale on left axis) and RRCBF (*dotted lines*, scale on right Y axis) dropped immediately to nonperfusing values on cardiac arrest (*shaded area*). Return of spontaneous circulation was followed by relative hypertension and ultimately a gradual return to baseline. RRCBF, in contrast, remained low after cardiac arrest and did not return to baseline within the 20-min follow-up period. There was no significant difference in MAP or RRCBF between 17β-estradiol-treated and vehicle-treated animals at any time point (mean ± SEM, n = 5 or 6/group; EST = estrogen treated; VEH = vehicle treated).

Table 2. Blood Analysis Data (Mean $\pm$ SEM) for Estradiol- *versus* Vehicle-treated Ovariectomized Wild-type Mice

Parameter	Vehicle (N = 5)	Estradiol (N = 5)
pH		
Baseline	7.3 ± 0.0	7.3 ± 0.0
5-min recovery	6.6 ± 0.0	6.6 ± 0.0
15-min recovery	6.8 ± 0.0	6.7 ± 0.1
$Paco_2$, mmHg		
Baseline	52 ± 2	47 ± 1
5-min recovery	78 ± 5	86 ± 4
15-min recovery	50 ± 4	51 ± 5
Pao_2, mmHg		
Baseline	286 ± 15	288 ± 15
5-min recovery	358 ± 32	281 ± 30
15-min recovery	462 ± 29	423 ± 56
Bicarbonate, mEq/L		
Baseline	21 ± 1	21 ± 1
5-min recovery	8.0 ± 0	7.7 ± 1
15-min recovery	7.0 ± 1	6.9 ± 1
Hemoglobin, mg/dL		
Baseline	14.1 ± 0.2	14.3 ± 0.2
5-min recovery	12.1 ± 0.4	11.7 ± 0.7
15-min recovery	11.8 ± 0.5	12.1 ± 0.7

Arterial blood analysis data from ovariectomized estrogen- and vehicle-treated mice. Arterial blood was obtained from the femoral artery in animals that underwent placement of arterial catheters but not cardiac arrest ("baseline"), and in animals that underwent cardiac arrest, 5 min after return of spontaneous circulation, and 15 min after return of spontaneous circulation. Values were similar for all parameters across groups. Data presented are mean $\pm$ SEM. SEMs of "0" and "0.0" resulted from rounding to appropriate significant digits.

$Paco_2$ = arterial carbon dioxide tension; Pao_2 = arterial oxygen tension.

Renal Ischemia–Reperfusion Injury after CA/CPR Is Sexually Dimorphic *In Vivo*

We then tested the hypothesis that the sex dimorphism found in human AKI and other animal models of renal ischemia is reproducible in our model. Histologic renal injury after CA/CPR is sexually dimorphic as seen in figures 3 and 4. The volume of necrotic tubular epithelium was $7 \pm 1\%$ in females as contrasted with $10 \pm 0\%$ in males (n = 10/group, $P = 0.04$). Females also exhibit protection from functional injury (BUN 65 ± 21 *vs.* 149 ± 27 mg/dl, $P = 0.04$; creatinine 0.14 ± 0.05 *vs.* 0.73 ± 0.16 mg/dl, $P = 0.01$, n = 6 males, 5 females).
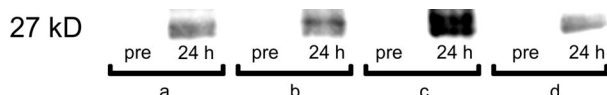
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Fig. 2. Urine neutrophil gelatinase-associated lipocalin is massively elevated 24 h after cardiac arrest/cardiopulmonary resuscitation (CA/CPR). Urine samples were obtained immediately before and 24 h after CA/CPR. A representative western blot is depicted. Small letters (a, b, c, and d) denote individual animals. pre = sample before cardiac arrest; 24 h = sample 24 h after cardiac arrest.
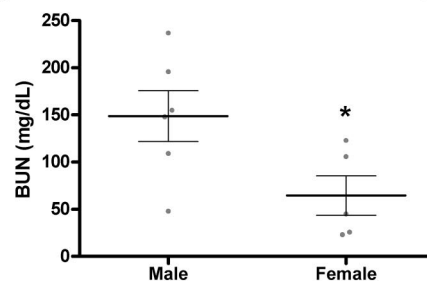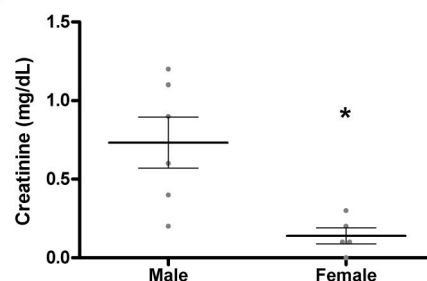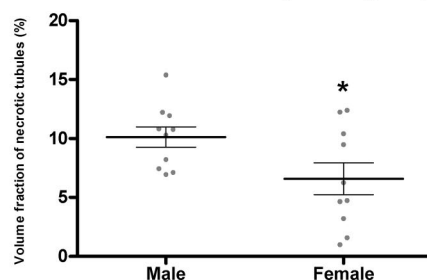

Fig. 3. Females are protected from functional and histolopathologic injury after cardiac arrest/cardiopulmonary resuscitation (CA/CPR). Blood and tissue samples were collected 24 h after CA/CPR. (*A* and *B*) Blood urea nitrogen (BUN) and creatinine. (*C*) Stereological assessment of volume of necrotic tubules as percent of total renal tissue volume (mean $\pm$ SEM, n = 10/group). * $P < 0.05$.

17β-estradiol Is Renoprotective in Whole Body Ischemia–Reperfusion

To establish whether the presence of estradiol protects against injury from global renal ischemia *in vivo*, gonadally intact females, vehicle-treated ovariectomized females, and estradiol-treated ovariectomized females were subjected to CA/CPR. The presence of 17β-estradiol, endogenous or exogenous, confers a reduction in renal functional and histopathologic injury (fig. 5). BUN was 65 ± 21, 166 ± 28, and 50 ± 14 mg/dl ($P = 0.002$); creatinine was 0.14 ± 0.05, 0.74 ± 0.26, and 0.23 ± 0.27 mg/dl ($P = 0.014$); and volume of necrotic tubular epithelium was $2.5 \pm 0.25\%$, $12.0 \pm 1.9\%$, and $5.0 \pm 1.6\%$ ($P = 0.004$) in intact females (n = 5), vehicle-treated ovariectomized females (n = 5), and estradiol-treated ovariectomized females (n = 8) respectively.

17β-estradiol Is Renoprotective in the Absence of ER-α or ER-β

ER-α and ER-β deficient animals along with WT littermate controls were treated with subcutaneous estradiol pellets
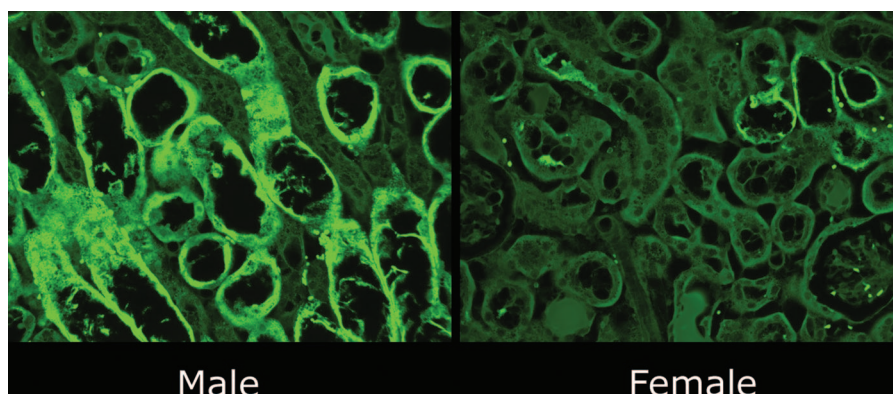
Fig. 4. Females are protected from histolopathologic injury after cardiac arrest/cardiopulmonary resuscitation. Representative photomicrographs of Flouro-Jade B-stained kidney sections from male and female mice. Flouro-Jade B stains dead cells bright green, in gray scale they appear bright white. Color version of this figure is available at www.anesthesiology.org.

or vehicle (ERαKO-estradiol, n = 5; ERαKO-vehicle, n = 5; ERβKO estradiol, n = 10; ERβKO-vehicle, n = 14; ERαWT-estradiol, n = 4; ERαWT-vehicle, n = 5; ERβWT-estradiol, n = 9; ERβWT-vehicle, n = 5) and subjected to CA/CPR to assess the receptor dependency of the renoprotective effect of 17β-estradiol (fig. 6). Estradiol reduced functional injury overall (BUN: estradiol 117 ± 71 *vs.* vehicle 167 ± 56, $P = 0.007$, creatinine: estradiol 0.5 ± 0.5 *vs.* vehicle 1.0 ± 0.4, $P = 0.013$); however, there was no significant difference in the amount of difference between estradiol- or vehicle-treated groups within strain/control groups. ER-α and ER-β gene-deleted mice both exhibited a nonsignificant tendency toward reduced BUN/creatinine when treated with estradiol. In histologic analysis, no significant effect of estradiol was found.

Because of the inconclusive finding of no difference in protection in the ER-α and ER-β gene-deleted mice, ovariectomized female mice treated with either 17β-estradiol or vehicle and ICI 182,780 (estradiol-ICI) also underwent CA/CPR. There was no difference in functional or histologic injury between the groups (BUN: estradiol 36 ± 9 *vs.* estradiol-ICI 27 ± 4, $P = 0.45$; creatinine: estradiol 0.3 ± 0 *vs.* estradiol-ICI 0.2 ± 0, $P = 0.62$; volume of necrotic tubules: estradiol 4.7 ± 1.1% *vs.* estradiol-ICI 4.7 ± 1.3%, $P = 0.97$, fig. 7).

Discussion

This study reports three important findings. First, females experience endogenous histologic and functional renoprotection after CA/CPR relative to their male counterparts. Second, loss of ovarian steroids results in enhanced renal injury, whereas estradiol replacement in ovariectomized females restores renal outcomes compared with that of the gonadally intact female. Third, beneficial effects of estradiol are not diminished by genetic deficiency of either subtype of the ER, or by blocking ER-α and ER-β. This finding indicates that estradiol-induced renoprotection after ischemia is not mediated through either of its cognate receptors. We conclude that estrogen-mediated renoprotection, likely act-

ing *via* a non-ER-mediated mechanism, is a significant component of the female advantage and sex dimorphism in whole body ischemia–reperfusion injury.

AKI is quite common after CA, occurring in up to 30% of survivors.[32] CA is itself an extreme example of whole body ischemia, which is a common precedent for AKI.[33] Diagnosis of AKI by serum creatinine is commonly delayed 24–48 h[34] because creatine kinase must be metabolized to creatinine to develop an elevated serum level. Active research into rapid biomarkers of AKI has generated a number of candidates, including NGAL, which is elevated in the urine within 2 h after focal ischemia[31] and reliably predicts AKI after cardiac surgery.[34,35] We have demonstrated in this study that NGAL is elevated in the urine 24 h after CA, suggesting that the CA/CPR model generates renal injury similar to that of human AKI with respect to the physiology that results in NGAL elevation. NGAL is likely elevated previously in the postarrest course, but our study only analyzed blood and urine at the 24-h time point.

The sexual dimorphism of renal ischemia–reperfusion injury, which we have reproduced here in the CA/CPR model, is well known and has been reviewed elsewhere.[36–38] However, the specific relative contribution of estrogen is a source of some contention, despite the fact that the benefit of estradiol to other organ systems has been well detailed.[15,39–45] The widely reported clinical observation that women have a decreased risk of AKI except after cardiac and vascular surgery[7,36,46–53] suggests that the clinically important mediator is estrogen (as women undergoing cardiac and vascular surgery are primarily postmenopausal). Indeed, Takaoka *et al.*[9] reported significant 17β-estradiol-induced reductions in BUN, creatinine, creatinine clearance, and urine output in rats subjected to 45 min of focal renal ischemia. Consistent with these results, Müller *et al.*[54] reported improved survival after renal ischemia in 17β-estradiol-treated male rats and a nonsignificant decrease in survival in ovariectomized females. However, they did not report renal function. In another focal ischemia model, Park *et al.*[8] reported that testosterone significantly worsens renal ischemia–reperfusion
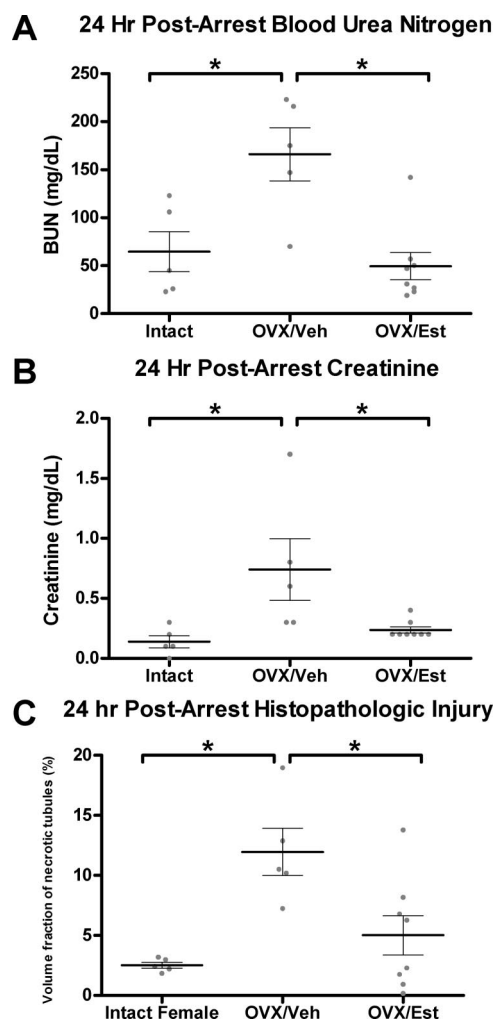
Fig. 5. Estrogen is renoprotective after cardiac arrest/cardiopulmonary resuscitation (CA/CPR). Endogenous estrogen (intact females, n = 5) or 17β-estradiol (n = 8) administration in ovariectomized (OVX) animals reduced functional and histopathologic renal injury when compared with vehicle (n = 8). Blood and tissue samples were collected 24 h after CA/CPR. (A and B) Blood urea nitrogen (BUN) and creatinine. (C) Stereological assessment of volume of necrotic tubules as percent of total renal tissue volume. Mean ± SEM, * P < 0.05.

Fig. 6. Estrogen is renoprotective, despite genetic deletion of estrogen receptors (ER). Female ER-α gene-deleted mice (ErαKO) and ER-β gene-deleted mice (ErβKO) were ovariectomized, treated with either 17β-estradiol or vehicle and compared with wild-type (WT) littermate controls (ErαWT and ErβWT, respectively). Although overall functional injury was significantly reduced in 17β-estradiol-treated animals, there is no significant difference in the magnitude of protection between any of the groups. Although all animals exhibited significant functional injury, the histopathologic injury in this cohort was not as profound as that in others, this may explain why the histopathologic protective effect was not present in any group. Blood and tissue samples were collected 24 h after cardiac arrest and cardiopulmonary resuscitation. (A and B) Blood urea nitrogen (BUN) and creatinine. (C) Stereological assessment of volume of necrotic tubules as percent of total renal tissue volume (mean ± SEM, ERβKO-vehicle n = 14, ERβKO-estradiol n = 10, ERβWT-vehicle n = 6, ERβWT-estradiol n = 11, ERαKO-vehicle n = 5, ERαKO-estradiol n = 5, ERαWT-vehicle n = 5, ERαWT-estradiol n = 4).

injury but that neither 17β-estradiol administration nor ovariectomy has as profound an effect.

Although our findings that ovariectomy significantly increases injury and that estradiol replacement restores protection support data from the study by Takaoka *et al.*,[9] they are not consistent with the results of the study by Park *et al.* Several important differences in the studies may account for this discrepancy. First, Takaoka *et al.*[9] used a higher dose of estradiol than Park *et al.*[8] (100 *vs.* 40 μg/kg), and the postmortem serum estradiol level in estradiol-treated animals in our study is twice that of the estradiol-treated animals in the study by Park *et al.* In fact, the anti-ischemic effect of 17β-estradiol has been shown to be dose dependent.[39] Second, the renal injury in the focal ischemic study by Park *et al.* is less profound than that induced by CA/CPR, that is, the mean BUN and creatinine are lower than our values. Indeed, in the
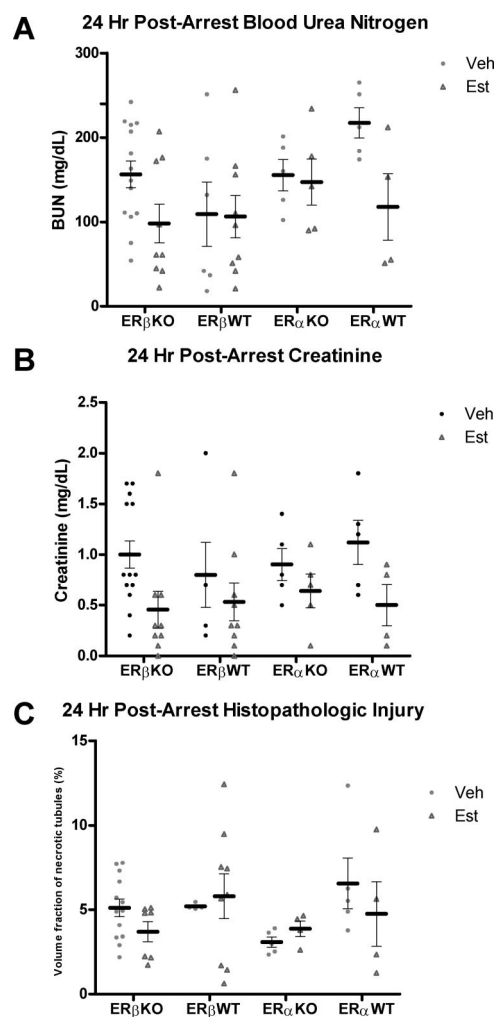
absence of testosterone, in general, there is little difference between ischemic and nonischemic animals in the study by Park *et al.* The renal insult from CA/CPR is profound as evidenced by the 10-fold increase in NGAL between pre- and postarrest states, and this alone may explain the difference in the magnitude of injury. Also, the focal ischemia model entails a large surgical field around the kidney, and local hypothermia may result, thus reducing injury. Occlusion of the

A

24 Hr Post-Arrest Blood Urea Nitrogen

B

24 Hr Post-Arrest Creatinine

C

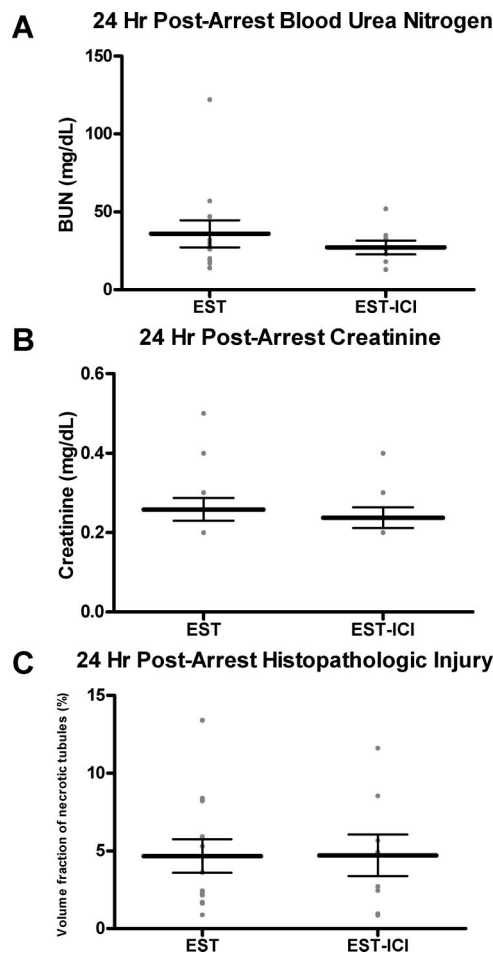### 24 Hr Post-Arrest Histopathologic Injury

Fig. 7. Estrogen is renoprotective after cardiac arrest/cardiopulmonary resuscitation (CA/CPR) in the presence of pharmacologic estrogen receptor (ER) blockade. Female C57BL/6 mice underwent ovariectomy and were treated with either 17β-estradiol (estradiol, n = 12) or 17β-estradiol and ICI 182,780 (estradiol-ICI, n = 8), a nonspecific ER antagonist before CA/CPR. Blood and tissue samples were collected 24 h after CA/CPR. There is no difference in functional or histopathologic injury between ER-free and ER-blockaded animals. (*A* and *B*) Blood urea nitrogen (BUN) and creatinine, respectively. (*C*) Stereological assessment of volume of necrotic tubules as percent of total renal tissue volume. Data presented are mean ± SEM.

entire renal pedicle involves the creation of venous and urinary congestion and may influence results. Although Park *et al.* and Takaoka *et al.* report maintaining animal temperatures near 37°C, these data have not been recorded in detail; therefore, it is unclear whether the renal tissue temperature was tightly controlled.[8,9]

The protection against ischemic effects conferred by 17β-estradiol is partly mediated *via* ERs in cardiac and cerebral ischemia, and indeed there is a receptor dimorphism in cerebral ischemia, with ER-α conferring protection in focal insults and ER-β acting in global ischemia.[55–57] Thus, it was reasonable to hypothesize that estrogen-mediated renoprotection might also be selectively mediated by one ER subtype. Our finding that ER-α and ER-β gene deletion or blockade does not alter the protective effect of estrogen suggests that estrogen-mediated

renoprotection occurs *via* a nonreceptor-mediated mechanism. This finding has important mechanistic and therapeutic implications: a novel mechanism may be at work in the kidney. Ischemia models in other tissues have demonstrated that protective effects of estrogen occur *via* phosphoinositide-3 kinase/Akt and p38 mitogen-activated protein kinase dependent up-regulation of heme oxygenase-1.[58,59] However, both the mechanisms have been shown to be dependent on classic ERs.[60,61] A receptor-independent action in renal ischemia may offer specific therapy for this organ system or imply that additional, previously unsuspected mechanisms function in addition to receptor-dependent mechanisms in other tissues. One possibility is that estradiol uses the so-called rapid membrane effects that are nongenomic in nature and involves in part cytosolic protein phosphorylation.[62] Some of these mechanisms may prove important in ischemia–reperfusion injury and offer important targets for further investigation and ultimately therapy. Recent investigation has highlighted the role of the novel g-protein-coupled ER, G-protein-coupled receptor 30,[63] which may mediate the effect we found in this experiment. A specific, nonestrogen agonist exists for this receptor that might offer far more specific effects than estrogen itself. Accordingly, further understanding of the mechanisms of estrogen-mediated renoprotection is vital if we are to harness the beneficial effects of estradiol and avoid undesirable consequences of the use of this pleiotropic steroid. Finally, estrogen regulation of nitric oxide and endothelin-1 has been shown to alter outcome of renal ischemia.[9,64] Because both these mediators are thought to act on renal blood flow, our finding that RRCBF is not affected by estrogen during the periarrest period suggests that these mechanisms are either not significantly regulated by estrogen in this model or not acutely regulated by estrogen in the periarrest period.

Our study has several limitations. CA/CPR is a severe physiologic challenge to the whole animal and includes whole body ischemia–reperfusion, unlike focal models of renal ischemia, for example, renal pedicle occlusion. In the CA/CPR model, it is impossible to exclude effects of distant organs on postarrest renal function. It is also unclear to what extent this model mimics clinical scenarios of prolonged perioperative hypotension or the no-flow period of suprarenal aortic clamping. We studied renal injury in mice but there may be profound differences between species in response to renal ischemia. Our evaluation of periarrest physiology is limited to arterial blood gases, mean arterial pressure, and RRCBF. We did not measure myocardial performance directly and cannot exclude a myocardial effect of estrogen leading to renal protection. RRCBF as measured by laser Doppler is specifically limited to a small superficial region of the kidney and may not reflect total renal blood flow. Because of the invasiveness of our measurement techniques and the susceptibility of laser Doppler to movement, we were unable to measure mean arterial pressure or RRCBF for more than 20 min after return of spontaneous circulation, thus we cannot exclude late effects of estrogen on renal blood flow. Estradiol-treated ERαKO mice required longer resuscitations than those receiving vehicle. It is possible that this

explains the loss of histologic protection specifically in this experimental group. However, these animals still exhibit reduced functional injury. Because equalization of the resuscitation time would be expected to increase injury in the vehicle arm or decrease injury in the estradiol arm, it is unlikely that this variation would change the conclusion that ER gene deletion does not alter the effect of estradiol. An effect of estrogen on histologic injury was not found in our testing of the effect of ER gene deletion. However, the level of both functional and histologic injury seen in this protocol is small and may have obscured real differences. Finally, we chose the 24-h time point to assess histopathology because in previous work functional injury was maximized at that time, and it is not possible for small rodents with severe AKI to survive after the 30 μl phlebotomy required. It is possible that 24 h is not the ideal time point to assess histopathology, and in future experiments, we plan to delineate the time course of injury in the CA/CPR model.

Because of limitations beyond our control, the number of ER gene-deleted mice was limited, particularly, the ErαWT-estradiol with n = 4. This low number of experimental animals makes it difficult to interpret the finding of lack of significant difference. Because of this doubt, we chose to confirm our interpretation of the data with the pharmacologic ER antagonist, ICI 182,780. We believe that the finding of no difference between estrogen-treated and estrogen/ICI 182,780-treated animals warrants our conclusion that the renoprotective effect of estradiol is not dependent on ER-α or ER-β. In general, sample sizes in this study are relatively small (range, 4–14/group), which might limit the strength of conclusions drawn from lack of statistically significant difference. However, the differences in renal insult between estrogen-treated and estrogen-deprived animals and between males and females are large in magnitude. This renders the lack of difference between ER intact and ER-deleted or blockaded animals all the more striking and suggests our conclusions are correct.

In summary, we have shown that estrogen is renoprotective in a global model of renal ischemia, namely, CA/CPR. Although it is premature to suggest the prophylaxis or treatment of renal ischemia with estrogens, the magnitude of the effect in our model offers promise for future therapy. We further present the first evidence to suggest a receptor-independent mechanism for this renoprotection. Our findings suggest that further investigation of estrogen-mediated renoprotection should focus on nongenomic actions of estrogen, and perhaps the novel ER, GPR30.

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