The Selective Estrogen Receptor Modulator, Raloxifene, Is Protective Against Renal Ischemia–Reperfusion Injury

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Background. There is increasing evidence that estrogen is responsible for improved outcomes in female kidney transplant recipients. Although the exact mechanism is not yet known, estrogen appears to exert its protective effects by ameliorating ischemia–reperfusion injury (IRI). In this study, we have examined whether the beneficial effects of exogenous estrogen in renal IRI are replicated by therapy with any one of several selective estrogen receptor modulators. **Methods.** C57BL/6 adult mice underwent standardized warm renal ischemia for 28 min after being injected with the selective estrogen receptor modulators, raloxifene, lasofoxifene, tamoxifen, bazedoxifene, or control vehicle (dimethyl sulfoxide), at 16 and 1 h before IRI. Plasma concentrations of blood urea nitrogen and creatinine were assessed 24, 48, 72, and 96 h post-IRI. Tissue was collected 30 d postischemia for fibrosis analysis using Sirius Red staining. **Results.** Raloxifene treatment in female mice resulted in significantly lower blood urea nitrogen and creatinine after IRI and significantly lower fibrosis 30 d following IRI. **Conclusions.** Raloxifene is protective against both acute kidney injury and fibrosis resulting from renal IRI in a mouse model.

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INTRODUCTION

There is an emerging body of evidence that estrogen is responsible for improved outcomes in female kidney transplant recipients.[1-4] Although the exact mechanism is not yet known, estrogen appears to exert its protective effects by ameliorating ischemia–reperfusion injury (IRI).[1,5-8] In renal transplantation IRI results in delayed graft function (DGF), defined as requiring dialysis in the first week after transplantation.[9] DGF results in increased costs, length of stay, readmissions after transplantation, rejection, and graft failure.[10-12] Any therapy that can ameliorate IRI and the attendant DGF will improve transplant outcomes and may enlarge the supply of organs by enabling use of organs with greater ischemic insult. We have previously demonstrated increased IRI tolerance in a mouse model with the administration of exogenous 17β-estradiol, and this has raised hopes of a potential therapy that might improve human deceased donor transplant outcomes[1]; however, there are known risks to systemic estrogen therapy, including increased risk of coronary artery disease, stroke, venous thromboembolism, and breast and endometrial cancer.[13,14] These risks have driven the development of selective estrogen receptor modulators (SERMs), a class of drugs that have tissue-specific agonist/antagonist actions on the estrogen receptor.[15] Clinically, SERMs are used for hormonal treatment/prevention of breast cancer, osteoporosis, and estrogen deficiency symptoms in postmenopausal women.[16-18] In this study, we have examined whether the beneficial effects of exogenous estrogen in renal IRI can be replicated by SERMs.

MATERIALS AND METHODS

Animals

We used 8- to 12-wk-old WT C57BL/6 adult female mice (The Jackson Laboratory) weighing 18 to 25 g.

Warm IRI Model

Mice were anesthetized with prewarmed pentobarbital sodium (65 mg/kg IP). Immediately after loss of a

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righting reflex, they were placed on a heated surgical pad (37 °C) in a temperature-controlled operative apparatus. Core body temperature was continuously measured throughout and maintained at 36.0 °C ± 0.5 °C. Using an operating microscope, an abdominal midline incision was made, and the left renal pedicle was exposed and clamped for 28 min with a microvascular clip (Roboz Surgical Instrument Co). Twenty-eight minutes was selected to yield significant IRI in control animals which was consistently survivable in the unilateral IRI and contralateral nephrectomy model in female mice.[19] For male mice, 15 min of clamp time yielded similar injury to 28 min in female mice.[1] After the clamp was released, the right kidney was exposed and removed, and the abdomen was closed. Animals were subcutaneously injected with 100 mL/kg warm saline after the operation to assist in the maintenance of hydration. Animals were kept in an incubator (37 °C) from the time of anesthetic administration until completely awake.[19]

SERMs

Raloxifene, lasofoxifene, tamoxifen, and bazedoxifene (Sigma Aldrich) were dissolved in dimethyl sulfoxide (4 mg/mL) and administered at a dose of 10 mg/kg at 16 and 1 h before administration of anesthesia for IRI. Control animals were injected with the carrier dimethyl sulfoxide (2.5 mL/kg) at 16 and 1 h before administration of anesthesia for IRI.

Blood Urea Nitrogen/Creatinine Measurements

Plasma concentrations of blood urea nitrogen (BUN) and creatinine were serially assessed 24, 48, 72, and 96 h post-IRI using an i-STAT Portable Clinical Analyzer with Chem8+ cartridges (Abbott Laboratories). These cartridges have a maximum BUN reading of 140 mg/dL and a minimum Cr reading of <0.2 mg/dL.

Tissue Collection and Histopathology

Under terminal general anesthesia, the left kidney in warm IRI experiments was harvested at 30 d after the ischemia experiment, fixed in 10% neutral-buffered formalin, and paraffin embedded. Histologic sections (4 μm) were stained with Sirius Red for quantification of fibrosis. Sirius Red–stained sections were scanned using the Aperio ScanScope CS slide scanner (Aperio Technologies, Leica Biosystems), and digitized images were analyzed with Aperio ImageScope software using an algorithm optimized for the detection of interstitial fibrosis (red staining of collagen fibers) as a percentage of the total volume of tissue on the slide.[19] Subcapsular areas were excluded so as to limit the analysis to parenchymal changes.

Statistics

BUN/creatinine curves were compared using 2-way ANOVA with Tukey's post hoc tests as appropriate. The Sirius Red staining percentage by group was compared pairwise using a 2-tailed Student *t* test. Statistical analysis was performed with GraphPad Prism, version 6.0h (GraphPad Software). A P of <0.05 was considered statistically significant.

Study Approval

All animal studies were approved by the IACUC of Children's Hospital of Philadelphia and performed at a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

RESULTS

Raloxifene-treated female animals showed significant protection from IRI. BUN was significantly lower in raloxifene-treated animals than in controls across all time points, and peak creatinine was significantly lower before returning to baseline at 72 h in both groups (Figure 1A and B). Sirius Red staining and automated quantification showed significantly less interstitial fibrosis in the raloxifene-treated animals than in controls (Figure 1C–G). Raloxifene treatment in male animals did not result in significant differences in BUN or creatinine (Figure 1H and I). Raloxifene treatment in female animals did not result in meaningful differences in baseline pre-IRI BUN or creatinine compared with controls (Figure 1J and K).

Lasofoxifene, tamoxifen, and bazedoxifene treatment did not appear to ameliorate IRI in this model. BUN and creatinine curves were not significantly different for any of these groups (Figure 2A–F). Based on the literature reports of tamoxifen's effect on renal interstitial fibrosis, Sirius Red staining was performed on the tamoxifen-treated animals. Tamoxifen-treated animals did not show significantly less fibrosis than controls (Figure 3).

DISCUSSION

We examined the effect of SERMs in a validated mouse model of renal IRI. Although prior work has demonstrated the protective influence of female sex and supplemental estrogen, no prior study has investigated the use of SERMs as a therapy to mitigate renal IRI. Our current work shows a marked protection from renal IRI with the use of raloxifene, with lower serum biomarkers of acute kidney injury and decreased fibrosis at 30 d. The classical description of estrogen receptor signaling involves the passive diffusion of lipophilic estrogen across cell membranes followed by interaction with the ligand binding domain of an estrogen receptor in either the cytoplasm or the nucleus. Binding 17β-estradiol leads to estrogen receptor dimerization, at which point the DNA binding domain of the dimerized estrogen receptor complex can bind to the targeted DNA motifs called estrogen response elements. There are 2 isoforms of the estrogen receptor, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Upon binding of estradiol, homo- or heterodimerization with either estrogen receptor is possible. Binding with an estrogen response element results in recruitment of transcriptional co-activators and ultimately gene transcription.[20] In addition to this classical pathway, estrogen and its receptors are also involved in nonnuclear signaling pathways, particularly in the endothelium, where estrogen is thought to increase endothelial NO synthase.[21]

We and other groups have demonstrated the protective role of supplemental 17β-estradiol in animal models of renal IRI, and we have demonstrated that the presence

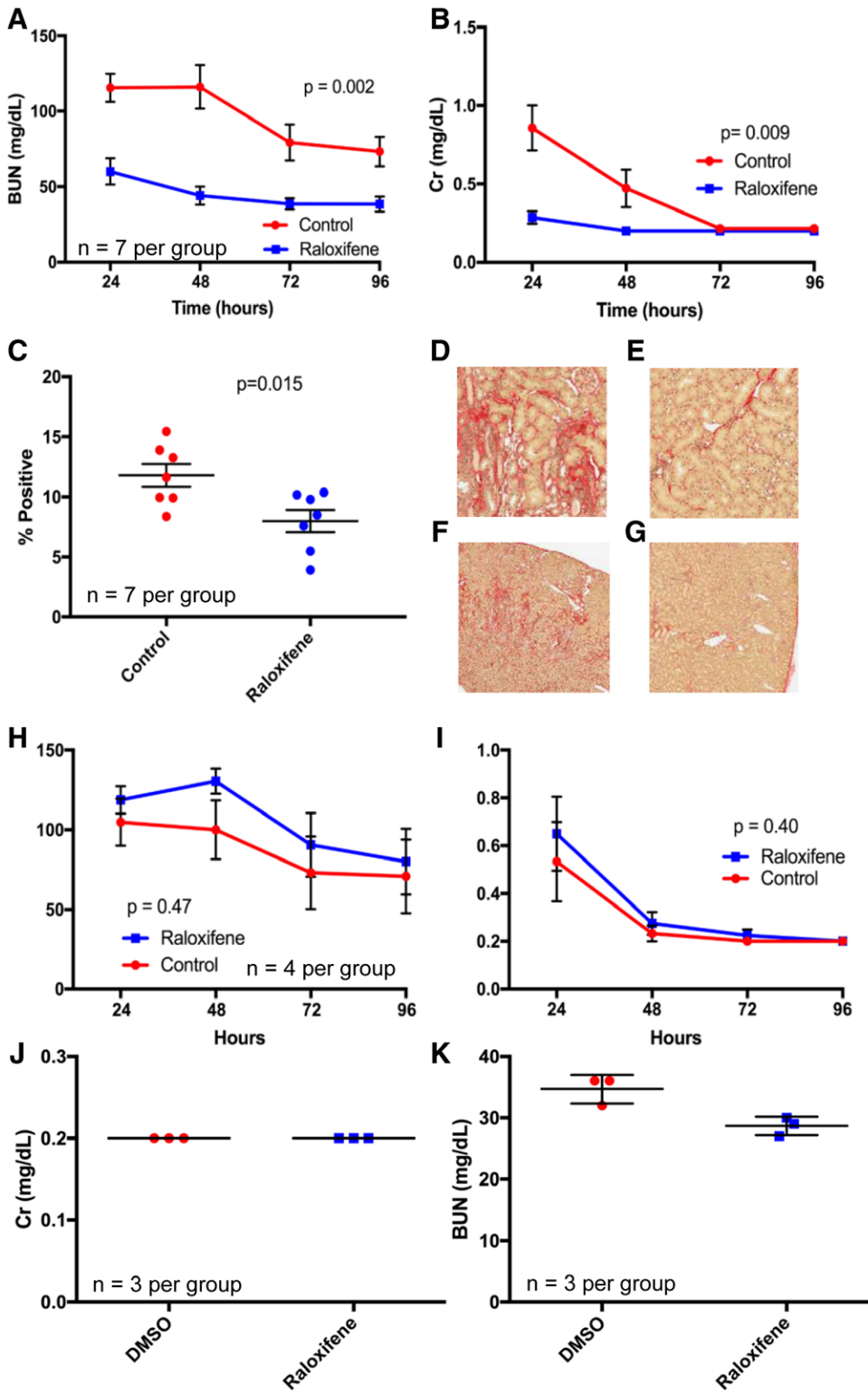

FIGURE 1. Female mice treated with raloxifene had significantly lower BUN (A) and creatinine (B) after IRI than controls; female mice treated with raloxifene had significantly less renal interstitial fibrosis at 30 d post-IRI by automated Sirius Red quantification (C). Representative images of Sirius Red staining of control (D and F) and raloxifene-treated animals (E and G) 30 d post-IRI. Male mice treated with raloxifene did not show a significant difference in BUN (H) or creatinine (I) post-IRI. Raloxifene did not meaningfully impact baseline BUN (J) or creatinine (K) levels in animals before IRI. BUN, blood urea nitrogen; IRI, ischemia–reperfusion injury.

of ERα is necessary for the improved renal IRI tolerance observed among female mice as well as the protective effect of supplemental 17β-estradiol.[1,5-8,22,23] Additionally, estrogen has also been investigated in cardiovascular disease and cardiac IRI[7,21]; however, large clinical studies have raised concerns that estrogen therapy may increase the risk of heart disease, venous thromboembolism, stroke, and breast cancer.[13,14]

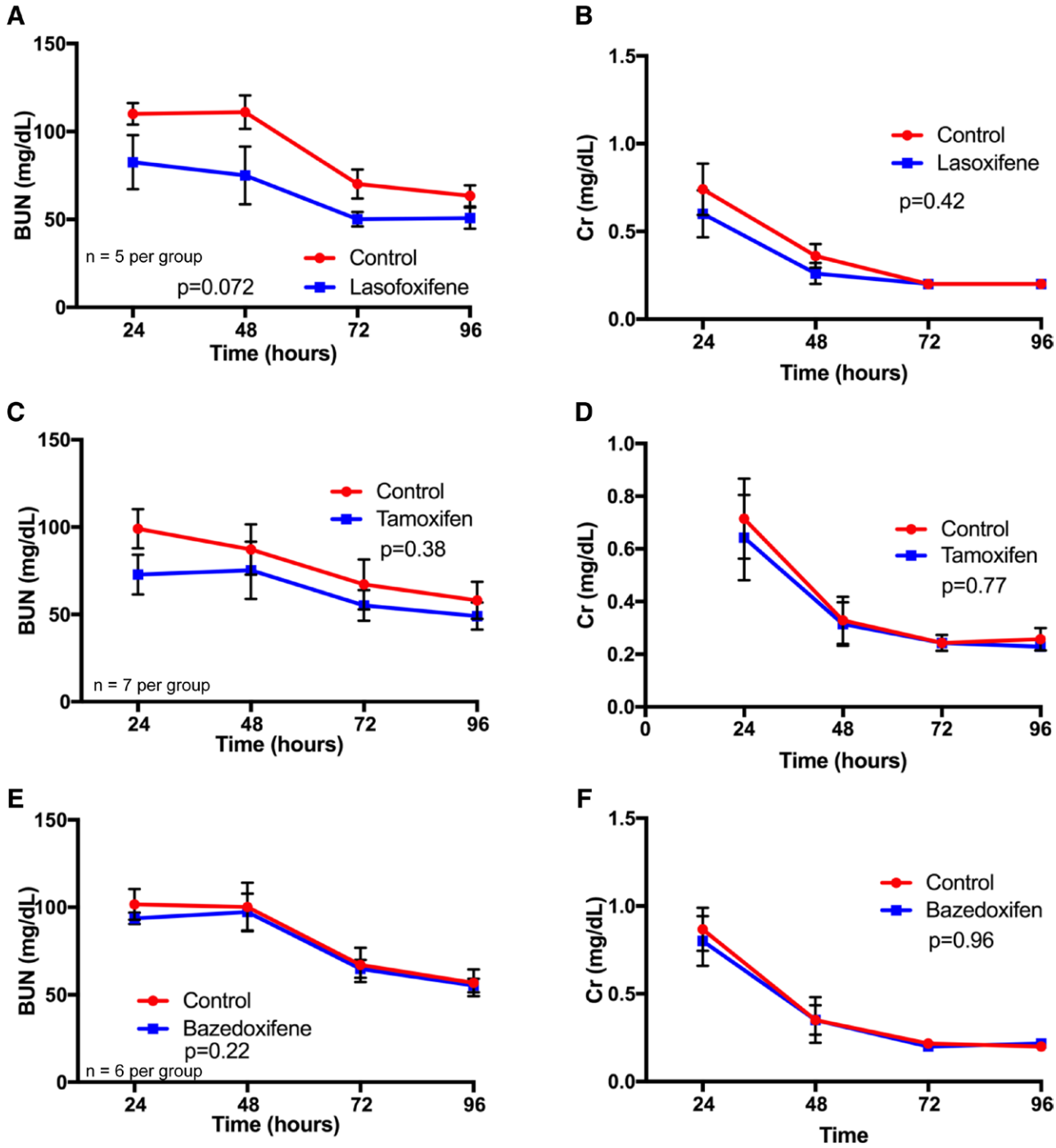

FIGURE 2. Female mice treated with lasofoxifene (A and B), tamoxifen (C and D), or bazedoxifene (E and F) did not show significantly less renal injury after warm IRI as measured by blood urea nitrogen or serum creatinine than controls. BUN, blood urea nitrogen; IRI, ischemia–reperfusion injury.

SERMs are a broad class of drugs with a variety of different tissue-specific agonist/antagonist effects on the estrogen receptor, and there is little knowledge of the effects of SERMs on renal IRI. Tamoxifen, the best known SERM, is in widespread clinical use in breast oncology, where its antagonist effect on the estrogen receptor helps to treat and prevent hormone responsive breast cancers. Unfortunately, tamoxifen has an agonist effect on the endometrium, resulting in an increased risk of endometrial cancers.[24] Other SERMs, such as raloxifene, are in clinical use for osteoporosis, where they function as estrogen receptor agonists on osteoblasts and are estrogen antagonists in breast tissue but lack tamoxifen's agonist effects on the endometrium.[25]

The tissue specificity of SERMs is under active investigation and likely is due to multiple factors. For example, the estrogen receptor is known to interact with a variety of different co-activators and co-repressors, which are expressed in a tissue-specific manner. Conformational changes to the estrogen receptor upon binding a SERM are thought to alter these interactions, resulting in differential effects based on the tissue-specific profile of co-activators and

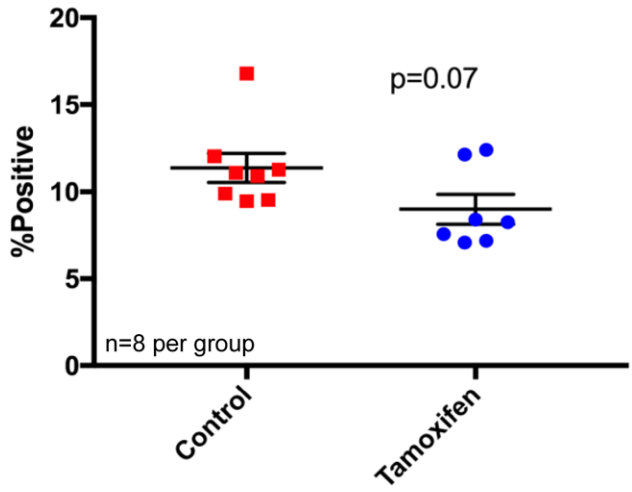

FIGURE 3. Female mice treated with tamoxifen did not show significantly less renal interstitial fibrosis at 30 d post-IRI by automated Sirius Red quantification. IRI, ischemia–reperfusion injury.

co-repressors.[15] Additionally, expression of ERα and ERβ varies by tissue type, and the SERMs have different affinities and effects on each receptor isoform.[26]

Although the effects of SERMs on breast cancer and bone density have been studied extensively, the effects of SERMs on IRI have yet to be elucidated. Animal studies have shown that raloxifene decreases IRI in a cardiac model.[27] Although no prior studies have specifically investigated the effect of SERMs in renal IRI, one study has shown that tamoxifen results in decreased fibrosis in a ureteral obstruction model of renal tubulointerstitial fibrosis.[28] In another study, raloxifene was shown to attenuate renal tubular damage in a hereditary glomerular nephritic model of renal injury.[29] Finally, a retrospective analysis of the raloxifene evaluation trial for osteoporosis showed slower increases in serum creatinine and slower decreases in estimated glomerular filtration rate in that cohort of postmenopausal women.[30]

There is a strong body of evidence that estrogen receptor-mediated signaling can ameliorate renal IRI and result in improved outcomes in transplantation and other disciplines. Additionally, several promising studies have suggested that SERMs may share some of estrogen's beneficial qualities with regard to IRI, without the off-target effects of estrogen; however, our study characterizes the effects of the SERMs in a validated mouse model of renal IRI, showing a clear protective effect of raloxifene and no benefit of other SERMs. This study further expands our understanding of the relationship between estrogen and IRI. Additionally, as raloxifene is safe and in widespread clinical use, this finding could rapidly be tested in the clinical environment, leading to new therapies to mitigate renal IRI.

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