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17β-ESTRADIOL ACCELERATED RENAL TUBULE REGENERATION IN MALE RATS AFTER ISCHEMIA/REPERFUSION-INDUCED ACUTE KIDNEY INJURY

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ABSTRACT—Ischemic/reperfusion injury (IRI) is the most common cause of acute kidney injury (AKI). Murine studies report that pretreatment with 17β-estradiol protects against AKI using multiple mechanisms, but how 17β-estradiol is involved in regenerating tubular cells is unknown. To visualize the kidney injury and repair, we used 17β-estradiol to treat rats with postischemic acute kidney injury. AKI was induced by clamping the renal pedicle for 90 minutes 2 weeks after a unilateral nephrectomy. Rats were treated with an intravenous injection of 17β-estradiol or vehicle immediately after reperfusion. Kidney injury was assessed by measuring biochemical and histopathological changes. Immunohistochemical staining of vimentin, proliferating cell nuclear antigen (PCNA), and E-cadherin were used to assess dedifferentiation, proliferation, and redifferentiation. Rats treated with 17β-estradiol had less kidney injury than did vehicle-treated rats post-IRI day 1. The number of PCNA-positive ($PCNA^{Pos}$) cells was significantly higher in post-IRI kidneys on day 1 in 17β-estradiol-treated rats. Moreover, $vimentin^{Pos}$ and $E\text{-}cadherin^{Pos}$ cells, which were interpreted as regeneration markers, were expressed earlier and significantly more copiously in 17β-estradiol-treated rats. We hypothesize that 17β-estradiol attenuates IRI-induced AKI by reducing inflammation and accelerating injured tubular cell regeneration.

KEYWORDS—Acute tubular necrosis, cell dedifferentiation, estrogen, renal proximal tubule, vimentin

INTRODUCTION

Acute kidney injury (AKI), characterized by increased blood levels of blood urea nitrogen (BUN) and creatinine (Cr), occurs in various clinical settings, including renal ischemia (1). One of the most common forms of human AKI is ischemia/reperfusion injury (IRI), which causes acute tubular injury. Ischemia leads to tubular cell damage, physiological dysfunction, and cell death, and reperfusion injures endothelial cells and then activates the overproduction of proinflammatory cytokines (2, 3). IRI models show that the kidney can recover from renal damage and dysfunction by repairing and regenerating renal tubular cells (4). Repairing the cells requires the dedifferentiation, proliferation, and migration of surviving renal tubular cells to replace dead cells after AKI (4).

Proliferating cell nuclear antigen (PCNA), a DNA polymerase δ-associated protein, is used as a marker for cell proliferation (5). PCNA-labeled nuclei identify a population of cells in the late G1 and S phases of the cell cycle (6). In contrast, vimentin (7), a mesenchymal cell marker, and E-cadherin, an epithelial cell marker (8, 9), have been used as markers of dedifferentiation and redifferentiation of the epithelial phenotype, and are interpreted together as regeneration. Vimentin expression usually becomes predominant on day 2 or 3 after reperfusion and is followed by E-cadherin expression (7, 8, 10). Sex differences in diseases have been reported (11–13). AKI is also less common and less severe in women than in men (14, 15). Estradiol is a female sex hormone that contributes the sex difference in diseases. Estradiol treatment protects organs against different injuries (16–18).

17β-estradiol (E2-β) prevents IRI-induced renal dysfunction and tissue injury in male rats (19, 20). In addition, estrogen protects the endothelial barrier function against IRI *in vitro* and *in vivo* (21). However, these three cited studies administered E2-β before inducing IRI, which does not mimic clinical conditions. It is not known whether E2-β accelerates tubular cell repair after IRI using the mechanisms involving regeneration (9). We therefore tested the hypothesis that E2-β is renoprotective in IRI by accelerating the regeneration of proximal tubular epithelial cells. Using IRI, which causes acute tubular injury in rats, we assessed cell proliferation (e.g., the number of PCNA-positive [$PCNA^{Pos}$] cells) and regeneration (e.g., the number of $vimentin^{Pos}$ and $E\text{-}cadherin^{Pos}$ cells) in IRI rats with and without E2-β treatment. We also evaluated renal function impairment, histopathologic outcomes, renal levels of a proinflammatory cytokine (e.g., tumor necrosis factor [TNF]-α) and an anti-inflammatory cytokine (e.g., interleukin [IL]-10), and serum levels of intercellular adhesion molecule 1 (ICAM1).

MATERIALS AND METHODS

All of the procedures in this project conformed to guidelines of the Department of Science and Technology of the Republic of China (Taipei, Taiwan) on

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Animal Care and were approved by the Animal Care Committee of Chi Mei Medical Center.

Animals and ischemia/reperfusion injury

Six-week-old male Sprague-Dawley rats were used in all IRI experiments. All of the rats were intraperitoneally (i.p.) injected with 25 mg/kg of the anesthetic tiletamine/zolazepam (Zoletil; Virbac, Carros, France) as an analgesic. After they had been randomly assigned to one of three groups—Sham Control (right nephrectomy and left kidney exposure but no IRI), Vehicle Control (right nephrectomy, left kidney exposure, IRI-induced AKI, and vehicle treatment), or 17β-Estradiol (right nephrectomy, left kidney exposure, IRI-induced AKI, and E2-β treatment) (six rats per group)—each rat's right kidney was removed through a small flank incision. After a 2-week recovery period, the rats were anesthetized again, and their left kidneys and renal vessels were exposed via a flank incision. The left renal artery was ligated with a small vascular clamp and released after 90 min. E2-β (5 mg/kg) (Sigma-Aldrich, St. Louis, MO) or vehicle (0.9% normal saline; 1 mL/kg) was injected (i.p.) immediately after reperfusion. In the Sham Control rats, the kidneys were treated identically, except for the clamping.

Rats exposed to 90 min of ischemia were housed in cages for 24 h after reperfusion. Blood samples were taken, the rats were overdosed with tiletamine/zolazepam, and then the left kidney was excised.

Renal function parameters

Both blood urea nitrogen (BUN) and creatinine (Cr) levels in plasma were determined using BUN and Cr assays (Wako Pure Chemicals, Osaka, Japan).

Histopathological evaluation

Excised left kidneys were processed, using standard procedures, for light-microscope examination. The kidneys were then fixed in phosphate-buffered 10% formalin, after which they were chopped into small pieces and embedded in paraffin wax, cut at 4 μm, and stained with periodic acid-Schiff stain. Histological changes were analyzed for tubular injury (22). Tubular necrosis and proteinaceous casts were graded as follows: no damage (0), mild (1: unicellular, patchy isolated damage), moderate (2: damage less than 25%), severe (3: damage between 25 and 50%), and very severe (4: more than 50% damage). The degree of medullary congestion was defined as follows: no congestion (0), mild (1: vascular congestion with erythrocytes identified using ×400 magnification), moderate (2: vascular congestion with erythrocytes identified using ×200 magnification), severe (3: vascular congestion with erythrocytes identified using ×100 magnification), and very severe (4: vascular congestion with erythrocytes identified using ×40 magnification). The histological data were scored by double-blinded independent observers.

Measuring inflammation response and endothelial cell damage

To evaluate the inflammatory response by checking IL-10 and TNF-α levels 24 h after reperfusion, kidney tissue was homogenized. The level of target proteins was measured using an enzyme-linked immunosorbent assay (ELISA) (Rat IL-10 DuoSet & Rat TNF-α DuoSet; R&D Systems, Minneapolis, MN). The severity of endothelial damage was evaluated by assaying serum samples obtained 24 h after reperfusion (Rat ICAM1 DuoSet; R&D Systems). Standard curves of study proteins provided in the ELISA kits were used to quantify the production of these proteins.

Quantifying apoptosis

Apoptotic cell death was quantified using an apoptosis assay (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI). Briefly, 3-μm-thick kidney sections were deparaffinized, hydrated, and treated for 10 min with proteinase K (PK) solution (20 mg/mL) in Tris-EDTA buffer (100 mM Tris-HCl [pH 8.0], 50 mM EDTA) with a final concentration of 20 μg/mL. After pre-equilibration, strands of DNA were end-labeled by incubating them with recombinant terminal deoxynucleotidyl transferase for 1 h at 37°C. The reaction was terminated by adding ×2 saline sodium citrate buffer for 15 min. After the nuclei had been washed, they were labeled with DAPI (Molecular Probes, Eugene, OR). The slides were then mounted and viewed at ×200 using a fluorescence microscope (Observer Z1; Carl Zeiss Microscopy, Munich, Germany). Apoptotic cells were counted in five randomly chosen nonoverlapping fields per section in the outer stripe of the renal medulla for each rat and are expressed as apoptotic cells per field.

Immunohistochemistry of proliferating cells

Proliferating cell nuclear antigen (PCNA) localization was detected on paraffin-embedded sections using the manufacturer's heat-induced epitope retrieval method (PCNA Staining Kit; Invitrogen, Carlsbad, CA). $PCNA^{Pos}$ cells were counted separately in nonoverlapping fields of each section of the outer stripe of the renal medulla (×200) of tubular areas for each rat and are expressed as the number of $PCNA^{Pos}$ cells per field in the tubular region.

Immunofluorescence and image analysis

Immunofluorescence staining was done using the manufacturer's instructions. The primary antibodies were used at the indicated dilutions: mouse monoclonal vimentin antibody (1:100) (ab8069; Abcam Plc, Cambridge, UK) and mouse monoclonal E-cadherin antibody (1:100) (ab76055; Abcam). Secondary antibodies used consisted of goat anti-mouse IgG H&L (1:200) (DyLight 594; ab96873; Abcam) for vimentin antibody, and goat anti-mouse IgG H&L (1:200) (DyLight 488; ab96871; Abcam) for E-cadherin antibody. Tissue sections were incubated with the primary antibody at 4°C overnight after they had been incubated with blocking solution (10% goat serum and 0.3% beef serum albumin [BSA] in PBS), and then the appropriate secondary antibody for 1 h at room temperature. The slides were mounted with Vectashield and examined using the fluorescence microscope.

The immunofluorescence staining was analyzed as described below: five fields from the outer stripe of the renal medulla were randomly selected and observed at ×200 magnification. The percentage (P) of positive stain area in each section was from 0 to 100%. The number of rats used is indicated for each experiment. Each immunostaining was done in duplicate.

Statistical analysis

SPSS 15.0 for Windows (SPSS Inc, Chicago, IL) was used for all analyses. Values are mean ± standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) and then Scheffé's test for multiple comparisons was used. Histological data were analyzed using the Kruskal–Wallis non-parametric test combined with Dunn multiple nonparametric pairwise test. Significance was set at $P < 0.05$.

RESULTS

Acute IRI was attenuated in E2-β-treated rats

An IRI model—90 min of ischemia and then 24 h of reperfusion—was used to induce AKI: severe tubular damage that resulted in pronounced renal dysfunction in the rats. A significant increase in the tubular necrosis score (Table 1) indicated tubular damage, and increased levels of plasma BUN and Cr (Fig. 1) indicated renal dysfunction. The renal damage score (Table 1) and plasma Cr level (Fig. 1) were significantly lower after E2-β immediate post-reperfusion treatment.

Cell regeneration was accelerated and cell apoptosis was attenuated in E2-β-treated IRI rats

Immunohistochemistry was used to analyze PCNA in postischemic kidneys to detect proliferating cells in proximal renal tubules 24 h after IRI had been induced (Fig. 2). To detect regeneration, immunofluorescence was used to analyze vimentin (Fig. 3) and E-cadherin (Fig. 4) in kidney epithelial cells

Table 1. **Renal damage scores in the rats 1 day after ischemia/reperfusion acute kidney injury**

Histopathological change	Sham Control	Vehicle Control	17β-estradiol	P^*
Tubular necrosis	0.32 ± 0.18	3.48 ± 0.10	2.77 ± 0.09	<0.01
Proteinaceous casts in tubules	0.17 ± 0.07	2.71 ± 0.42	2.12 ± 0.26	0.35
Medullary congestion	0.69 ± 0.05	3.02 ± 0.22	2.56 ± 0.24	0.12

*17β-estradiol group compared with Vehicle-Control group.

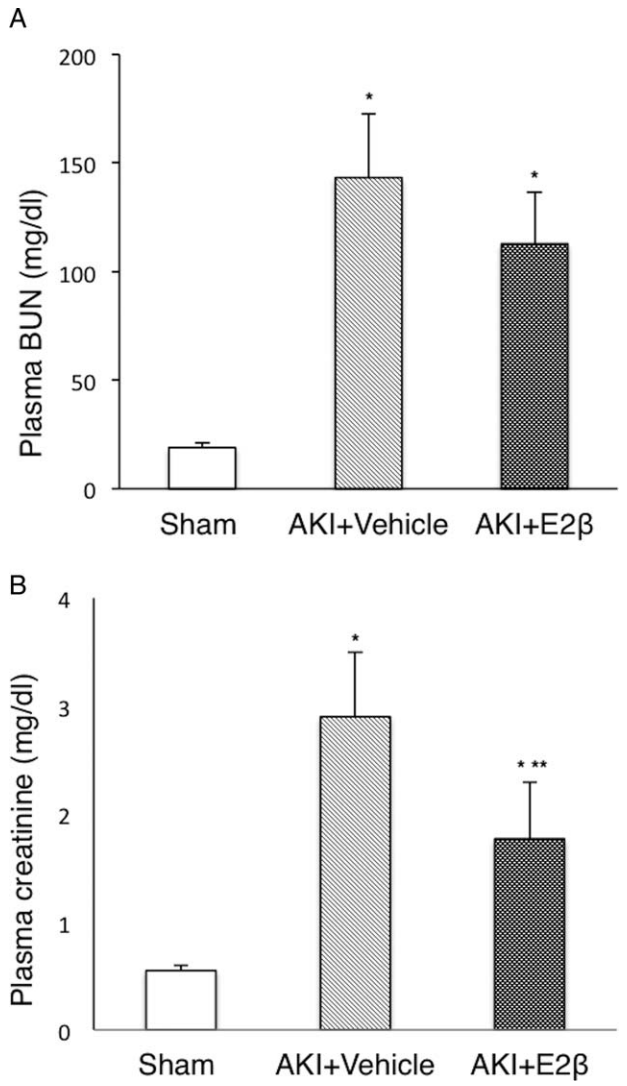

Fig. 1. **Effect of 17β-estradiol on blood urea nitrogen (BUN) (A) and plasma creatinine concentration (B) 1 day after reperfusion.** Vehicle or 17β-estradiol (5 mg/kg) was intravenously (i.v.) injected immediately after reperfusion. Each column and bar represents the mean ± S.E.M. (n = 6). $^{*}P < 0.05$, compared with Sham Controls. $^{**}P < 0.05$, compared with Vehicle Controls. AKI indicates acute kidney injury; S.E.M.; standard error of the mean.

24 h after ischemia. TUNEL assays were used to detect apoptotic cell death 24 h after ischemia (Fig. 5). As expected, there were more PCNA^{Pos} cells both in Vehicle Control and in E2-β-treated IRI rats, and there were significantly more in E2-β-treated rats than in Vehicle Control and Sham Control rats. There were significantly more Vimentin^{Pos} and E-cadherin^{Pos} cells in E2-β-treated IRI rats than in Sham Controls and Vehicle Controls (Figs. 2–4). In contrast, there were significantly fewer TUNEL^{Pos} cells in E2-β-treated AKI rats than in Vehicle Controls and Sham Controls (Fig. 5).

Overproduction of several systemic inflammatory response molecules was downregulated in E2-β-treated IRI rats

To detect endothelial damage in ischemic kidneys, renal levels of TNF-α and IL-10 and serum levels of ICAM1 were determined in IRI rats 24 h postischemia (Fig. 6). Again, as expected, TNF-α and ICAM1 levels were significantly lower and the IL-10 level was significantly higher in E2-β-treated IRI rats than in Vehicle Controls (Fig. 6).

DISCUSSION

We found that intravenously injecting rats with E2-β immediately after IRI attenuated their functional impairment by reducing apoptosis and inflammation, increasing endothelial cell survival, and accelerating renal regeneration. The expression of cell adhesion molecules such as ICAM1 on damaged endothelial cells and the production of cytokines increased, which is consistent with other reports (23, 24) that endothelial cells contribute to the pathology of ischemic AKI in rats. Chronic kidney fibrosis after IRI is associated with the loss of peritubular capillaries and the severity of decreased peritubular perfusion in animal experiments (25). E2-β induces endothelial cell proliferation and migration mediated by the classic estrogen receptor, which is expressed by endothelial cells (26). Thus, the renoprotective effects of E2-β might be related to the restoration of normal endothelial function in renal ischemia. E2-β protects against renal ischemic injury in several ways: it attenuates renal ischemic injury after cardiac arrest by reducing postischemic glomerular endothelial hyperpermeability (21); it decreases the production of renal endothelin-1 from

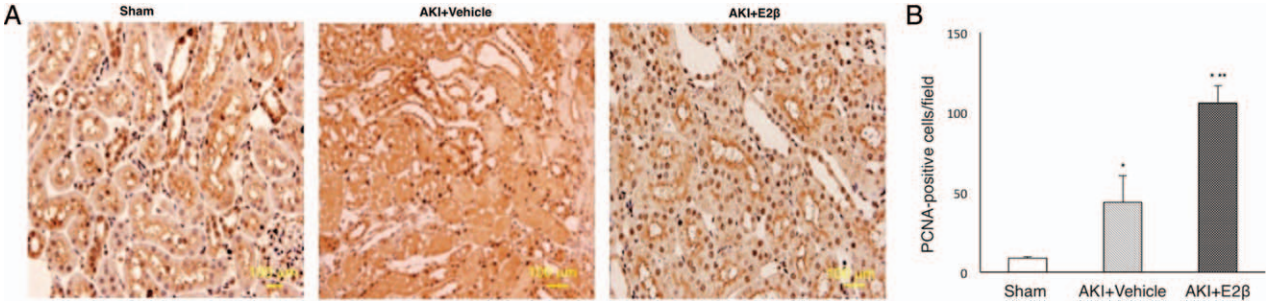

Fig. 2. **A, Light microscopy of the renal tubules of a Sham Control rat, a Vehicle Control rat, and a 17β-Estradiol AKI rat.** 17β-estradiol (5 mg/kg) was injected immediately after reperfusion. Proliferating cell nuclear antigen (PCNA) immunoperoxidase staining, original magnification ×200. B, Each column and bar represents the means ± S.E.M. (n = 6). $^{*}P < 0.05$, compared with Sham Controls. $^{**}P < 0.05$, compared with Vehicle Controls. [See previous figure legend for abbreviations.]

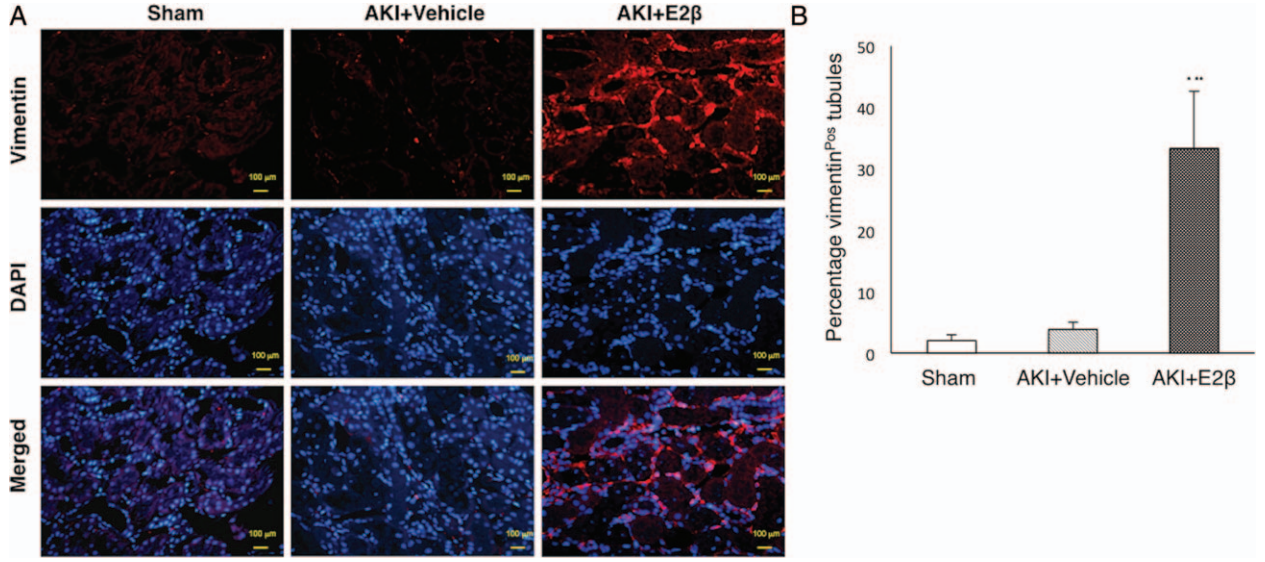

FIG. 3. **A, Colocalization of vimentin and DAPI in kidney cells 24 h after reperfusion of a Sham Control rat, a Vehicle Control AKI rat, and a 17β-Estradiol AKI rat.** Immunofluorescence staining, original magnification ×200. B, Each column and bar represents the mean ± S.E.M. (n = 6). $^{*}P < 0.05$, compared with Sham Controls, $^{**}P < 0.05$, compared with Vehicle Controls. [See previous figure legends for abbreviations.]

injured endothelial cells after IRI in male rats (20); it activates the PI3k/Akt/eNOS pathway (22); and it stimulates bone marrow-derived endothelial progenitor cells to migrate to ischemic tissue to promote tissue repair by increasing angiogenesis (27, 28).

The marked increase in PCNA^{Pos} cells in renal proximal tubules as early as 1 day after IRI indicates the initiation of a repair process; this increase was even greater in E2-β-treated rats. Vimentin, a mesenchymal marker, is usually expressed only in the early stages of kidney development. It can, however, be expressed in proximal renal tubules after IRI, which indicates that these cells are dedifferentiating for renal repair (7, 29). The expression of vimentin becomes apparent on day 2 or 3 after IRI, but our findings of significantly more vimentin^{Pos} cells 1 day after IRI in E2-β-treated rats indicate the acceleration of the regeneration and repair process. E-cadherin, a transmembrane glycoprotein, is essential for maintaining epithelial polarity as well as for establishing tight junctions (30). E-cadherin is present only at very low levels in proximal renal tubules in rats without IRI, but it can increase after IRI (8, 31). The expression of E-cadherin after IRI usually appears after vimentin expression, which indicates the redifferentiation of tubular cells. We found that E-cadherin expression was also detectable on day 1 post-IRI in E2-β-treated rats, but not in vehicle-treated rats. The marked increase in PCNA^{Pos} cells, vimentin^{Pos} cells, and E-cadherin^{Pos} cells in renal tubules as

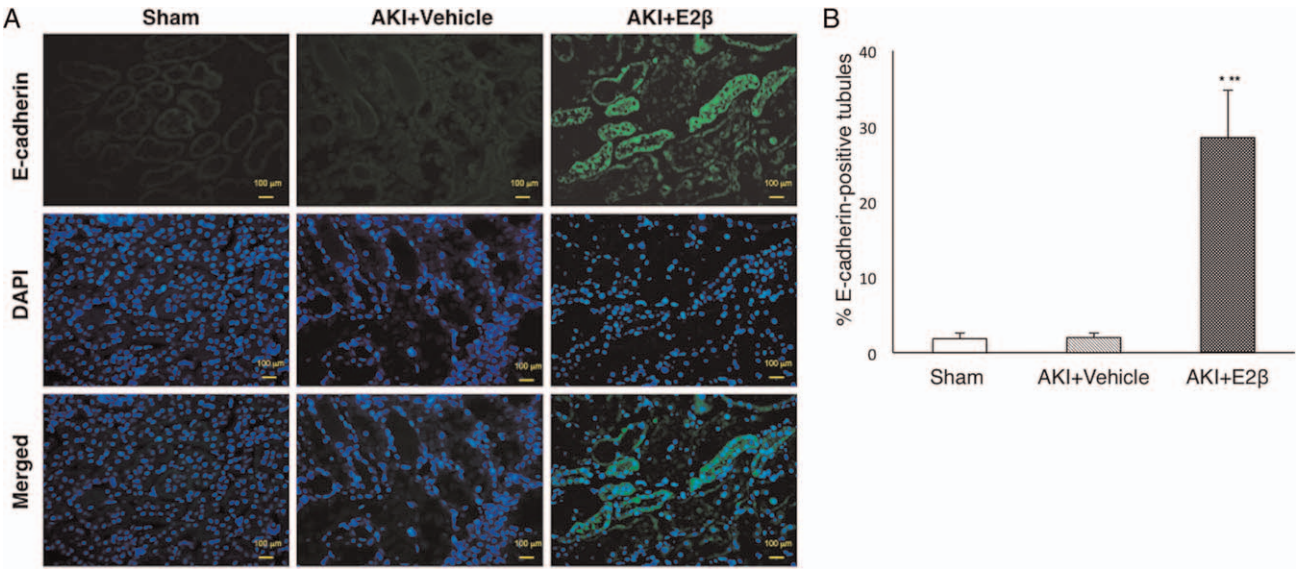

FIG. 4. **A, Colocalization of E-cadherin and DAPI 24 h post-reperfusion in the kidney of a Sham Control rat, a Vehicle Control AKI rat, and a 17β-Estradiol AKI rat.** Immunofluorescence staining, original magnification ×200. B, Each column and bar represents the mean ± S.E.M. (n = 6). $^{*}P < 0.05$, compared with Sham Controls, $^{**}P < 0.05$, compared with Vehicle Controls. [See previous figure legends for abbreviations.]

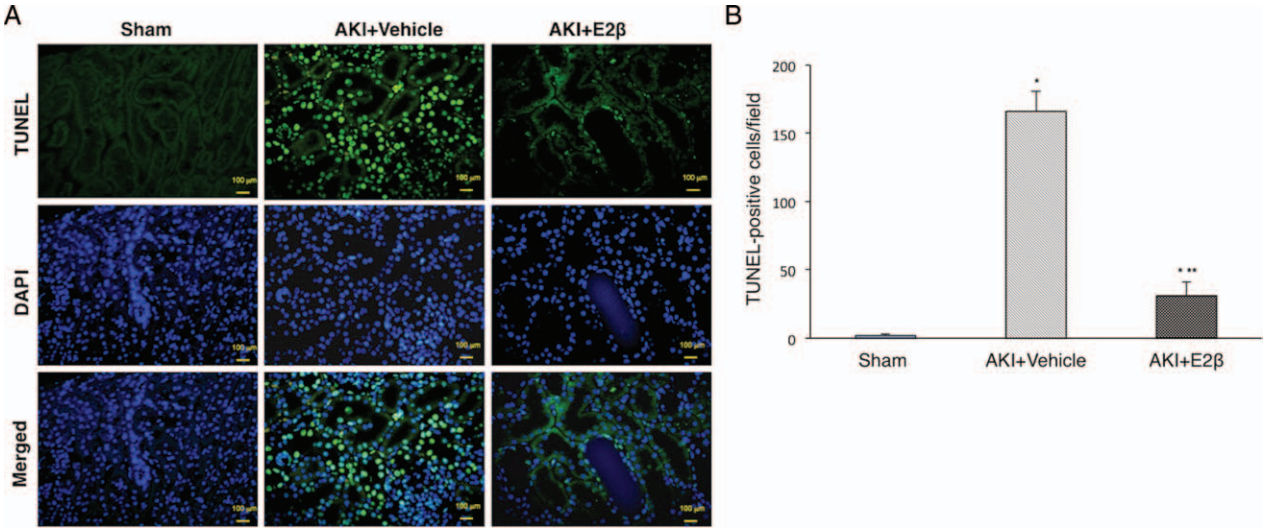

FIG. 5. **A, Colocalization of TUNEL and DAPI in kidney cells at 24 h post-reperfusion of a Sham Control rat, a Vehicle Control AKI rat, and a 17β-Estradiol AKI rat.** Immunofluorescence staining, original magnification ×200. B, Each column and bar represents the mean ± S.E.M. (n = 6). $^{*}P < 0.05$, compared with Sham Controls, $^{**}P < 0.05$, compared with Vehicle Controls. [See previous figure legends for abbreviations.]

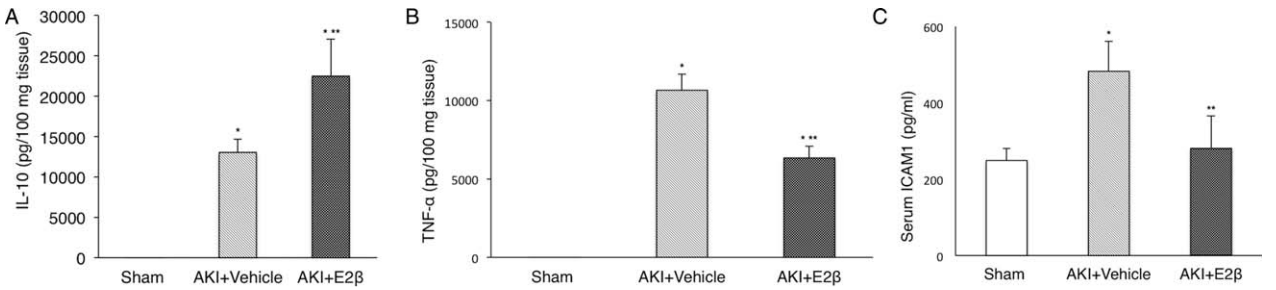

FIG. 6. **Levels of both IL-10 (A) and TNF-α (B) in the kidney homogenates, and the serum levels of ICAM1 (C) for Sham Controls, Vehicle Control AKI rats, and 17β-Estradiol AKI rats.** Each column and bar represents the mean ± S.E.M. (n = 6). $^{*}P < 0.05$, compared with Sham Controls, $^{**}P < 0.05$, compared with Vehicle Controls. [See previous figure legends for abbreviations.]

early as 1 day after IRI in E2-β-treated rats suggests that the surviving tubular cells accelerate the process of regeneration. E2-β increases neural stem cell differentiation and proliferation, and it regulates skeletal muscle differentiation *in vitro* (32–35). Recent studies (36, 37) have also shown that E2-β promotes the osteogenic differentiation of mesenchymal stem cells *in vitro*. These findings imply that E2-β accelerates kidney regeneration.

Perhaps our most striking finding was that E2-β attenuated the impairment of renal function during IRI by promoting cell proliferation, accelerating cell regeneration, and reducing inflammation after IRI.

This study had some limitations. We administered E2-β immediately after IRI induction; therefore, we do not know whether we can detect faster regeneration if we inject E2-β at a later time point when the level of inflammation is greater. We also do not know whether the faster renal proximal tubular regeneration is because of the direct effect of E2-β or because of the indirect effects of the anti-inflammation and endothelial cell protection of E2-β. Moreover, we did not study the molecular mechanism behind the ability of E2-β to accelerate renal tubular cell regeneration. Additional studies are warranted for these questions.

In summary, this is the first study to show that E2-β had a beneficial effect on postischemic kidneys during the repair phase by accelerating the regeneration process. The pleiotropic effect of E2-β might serve as a therapeutic approach for treating AKI.

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REFERENCES

1. Thadhani R, Pascual M, Bonventre JV: Acute renal failure. *N Engl J Med* 334(22):1448–1460, 1996.
2. Bonventre JV, Zuk A: Ischemic acute renal failure: an inflammatory disease? *Kidney Int* 66(2):480–485, 2004.
3. Gill N, Nally JV Jr, Fatica RA: Renal failure secondary to acute tubular necrosis: epidemiology, diagnosis, and management. *Chest* 128(4):2847–2863, 2005.
4. Humphreys BD, Czerniak S, DiRocco DP, Hasnain W, Cheema R, Bonventre JV: Repair of injured proximal tubule does not involve specialized progenitors. *Proc Natl Acad Sci U S A* 108(22):9226–9231, 2001.

5. Bonventre JV, Leaf A, Malis CD: Nature of the cellular insult in ischemic acute renal failure. In: Brenner BM, Lazarus JM, editors. Acute Renal Failure. New York: Churchill-Livingstone; 1998. pp. 3–43.
6. Connolly KM, Bogdanffy MS: Evaluation of proliferating cell nuclear antigen (PCNA) as an endogenous marker of cell proliferation in rat liver: a dual-stain comparison with 5-bromo-2′-deoxyuridine. *J Histochem Cytochem* 41(1):1–6, 1993.
7. Witzgall R, Brown D, Schwarz C, Bonventre JV: Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogenous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. *J Clin Invest* 93(5):2175–2188, 1994.
8. Maeshima A, Yamashita S, Nojima Y: Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney. *J Am Soc Nephrol* 14(12):3138–3146, 2003.
9. Matsuda H, Lavoie JL, Gaboury L, Hamet P, Tremblay J: HCaRG accelerates tubular repair after ischemic kidney injury. *J Am Soc Nephrol* 22(11):2077–2089, 2011.
10. Fujigaki Y, Sakakima M, Sun Y, Fujikura T, Tsuji T, Yasuda H, Hishida A: Cell division and phenotypic regression of proximal tubular cells in response to uranyl acetate insult in rats. *Nephrol Dial Transplant* 24(9):2686–2692, 2009.
11. Ngo ST, Steyn FJ, McCombe PA: Gender differences in autoimmune disease. *Front Neuroendocrinol* 35(3):347–369, 2014.
12. Maas AH, Appelman YE: Gender differences in coronary heart disease. *Neth Heart J* 18(12):598–602, 2010.
13. Angele MK, Schwacha MG, Ayala A, Chaudry IH: Effect of gender and sex hormones on immune responses following shock. *Shock* 14(2):81–90, 2000.
14. Wei Q, Wang MH, Dong Z: Differential gender differences in ischemic and nephrotoxic acute renal failure. *Am J Nephrol* 25(5):491–499, 2005.
15. Xue JL, Daniels F, Star RA, Kimmel PL, Eggers PW, Molitoris BA, Himmelfarb J, Collins AJ: Incidence and mortality of acute renal failure in Medicare beneficiaries, 1992 to 2001. *J Am Soc Nephrol* 17(4):1135–1142, 2006.
16. Breithaupt-Faloppa AC, Fantozzi ET, de Assis Ramos MM, Vitoretti LB, Couto GK, Lino-dos-Santos-Franco A, Rossoni LV, Oliveira-Filho RM, Vargaftig BB, Tavares-de-Lima W: Protective effect of estradiol on acute lung inflammation induced by an intestinal ischemic insult is dependent on nitric oxide. *Shock* 40(3):203–209, 2013.
17. Cuzzocrea S, Genovese T, Mazzon E, Esposito E, Di Paola R, Muià C, Crisafulli C, Peli A, Bramanti P, Chaudry IH: Effect of 17beta-estradiol on signal transduction pathways and secondary damage in experimental spinal cord trauma. *Shock* 29(3):362–371, 2008.
18. Lin FS, Shen SQ, Chen ZB, Yan RC: 17beta-estradiol attenuates reduced-size hepatic ischemia/reperfusion injury by inhibition apoptosis via mitochondrial pathway in rats. *Shock* 37(2):183–190, 2012.
19. Hutchens MP, Nakano T, Kosaka Y, Dunlap J, Zhang W, Herson PS, Murphy SJ, Anderson S, Hurn PD: Estrogen is renoprotective via a nonreceptor-dependent mechanism after cardiac arrest in vivo. *Anesthesiology* 112(2):395–405, 2010.
20. Takaoka M, Yuba M, Fujii T, Ohkita M, Matsumura Y: Oestrogen protects against ischaemic acute renal failure in rats by suppressing renal endothelin-1 overproduction. *Clin Sci (Lond)* 48:434S–437S, 2002.
21. Hutchens MP, Fujiyoshi T, Komers R, Herson PS, Anderson S: Estrogen protects renal endothelial barrier function from ischemia-reperfusion in vitro and in vivo. *Am J Physiol Renal Physiol* 303(3):F377–F385, 2012.
22. Satake A, Takaoka M, Nishikawa M, Yuba M, Shibata Y, Okumura K, Kitano K, Tsutsui H, Fujii K, Kobuchi S, et al.: Protective effect of 17beta-estradiol on ischemic acute renal failure through the PI3K/Akt/eNOS pathway. *Kidney Int* 73(3):308–317, 2008.
23. Basile DP, Friedrich JL, Spahic J, Knipe N, Mang H, Leonard EC, Changizi-Ashtiyani S, Bacallao RL, Molitoris BA, Sutton TA: Impaired endothelial proliferation and mesenchymal transition contribute to vascular rarefaction following acute kidney injury. *Am J Physiol Renal Physiol* 300(3):F721–F733, 2011.
24. Rabelink TJ, de Boer HC, van Zonneveld AJ: Endothelial activation and circulating markers of endothelial activation in kidney disease. *Nat Rev Nephrol* 6(7):404–414, 2010.
25. Kramann R, Tanaka M, Humphreys BD: Fluorescence microangiography for quantitative assessment of peritubular capillary changes after AKI in mice. *J Am Soc Nephrol* 25(9):1924–1931, 2014.
26. Venkov CD, Rankin AB, Vaughan DE: Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. *Circulation* 94(4):727–733, 1996.
27. Zhang JC, Lü G: Effect of 17β-estradiol in rat bone marrow-derived endothelial progenitor cells. *Mol Med Rep* 8(1):178–182, 2013.
28. Zhao X, Huang L, Yin Y, Fang Y, Zhao J, Chen J: Estrogen induces endothelial progenitor cells proliferation and migration by estrogen receptors and PI3K-dependent pathways. *Microvasc Res* 75(1):45–52, 2008.
29. Holthöfer H, Miettinen A, Lehto VP, Lehtonen E, Virtanen I: Expression of vimentin and cytokeratin types of intermediate filament proteins in developing and adult human kidneys. *Lab Invest* 50(5):552–559, 1984.
30. Tunggal JA, Helfrich I, Schmitz A, Schwarz H, Günzel D, Fromm M, Kemler R, Krieg T, Niessen CM: E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *EMBO J* 24(6):1146–1156, 2005.
31. Prozialeck WC, Lamar PC, Appelt DM: Differential expression of E-cadherin, N-cadherin and beta-catenin in proximal and distal segments of the rat nephron. *BMC Physiol* 17(4):10, 2004.
32. Brännvall K, Korhonen L, Lindholm D: Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. *Mol Cell Neurosci* 21(3):512–520, 2002.
33. Kishi Y, Takahashi J, Koyanagi M, Morizane A, Okamoto Y, Horiguchi S, Tashiro K, Honjo T, Fujii S, Hashimoto N: Estrogen promotes differentiation and survival of dopaminergic neurons derived from human neural stem cells. *J Neurosci Res* 79(3):279–286, 2005.
34. Galluzzo P, Rastelli C, Bulzomi P, Acconcia F, Pallottini V, Marino M: 17beta-Estradiol regulates the first steps of skeletal muscle cell differentiation via ER-alpha-mediated signals. *Am J Physiol Cell Physiol* 297(5):C1249–C1262, 2009.
35. Ogawa M, Yamaji R, Higashimura Y, Harada N, Ashida H, Nakano Y, Inui H: 17β-estradiol represses myogenic differentiation by increasing ubiquitin-specific peptidase 19 through estrogen receptor α. *J Biol Chem* 286(48): 41455–41465, 2011.
36. Irmak G, Demirtaş TT, Cetin Altindal D, Calışand M, Gümüşderelioğlu M: Sustained Release of 17β-estradiol stimulates osteogenic differentiation of adipose tissue-derived mesenchymal stem cells on chitosan-hydroxyapatite scaffolds. *Cells Tissues Organs* 199(1):37–50, 2014.
37. Qiu X, Jin X, Shao Z, Zhao X: 17β-estradiol induces the proliferation of hematopoietic stem cells by promoting the osteogenic differentiation of mesenchymal stem cells. *Tohoku J Exp Med* 233(2):141–148, 2014.