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ORIGINAL ARTICLE

The role of Sigma-1 receptor in sex-specific heat shock response in an experimental rat model of renal ischaemia/reperfusion injury

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SUMMARY

We previously showed that female rats are more protected against renal ischaemia/reperfusion (I/R) injury than males, which is partly attributed to their more pronounced heat shock response. We recently described that Sigma-1 receptor (S1R) activation improves postischaemic survival and renal function. 17β-estradiol activates S1R, thus here we investigated the role of sex-specific S1R activation and heat shock response in severe renal I/R injury. Proximal tubular cells were treated with 17β-estradiol, which caused direct S1R activation and subsequent induction of heat shock response. Uninephrectomized female, male and ovariectomized female (Ovx) Wistar rats were subjected to 50-min renal ischaemia followed by 2 (T2) and 24 (T24) hours of reperfusion. At T24 renal functional, impairment was less severe and structural damage was less prominent in females versus males or Ovx. Postischaemic increase in S1R, pAkt, HSF-1, HSP72 levels were detected as early as at T2, while pHSP27 was elevated later at T24. Abundance of heat shock proteins was higher in healthy female rats and remained higher at T2 and T24 (female versus male or Ovx; resp.). We propose a S1R-dependent mechanism, which contributes to the relative renoprotection of females after I/R injury by enhancing the heat shock response.

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Key words
heat shock proteins, heat shock response, kidney ischaemia/reperfusion, oestrogen, sex differences, Sigma-1 receptor

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Introduction

Morbidity in many ischaemia-related diseases such as myocardial infarction or stroke is higher in men than in women. However, after menopause, the incidence of ischaemic disease is similar in both sexes and women may even have accelerated atherosclerosis due to excessive oestrogen loss [1]. Sex differences also exist in the susceptibility to ischaemia/reperfusion (I/R) injury of the kidney. We previously showed that the outcome of ischaemia-induced acute kidney injury (AKI) is substantially improved in female rats; they have better postischaemic survival rates and renal function than males [2].

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The molecular mechanism of I/R injury-induced AKI is clearly multifactorial and is still not fully understood. Kidney hypoperfusion caused by systemic and local renal vasoconstriction is a major factor leading to I/R injury. We demonstrated that the expression of vasoconstrictor endothelin-1 is higher in male rats, which together with impaired nitric oxide synthase (NOS) expression and activity plays a significant part in their worse postischaemic renal perfusion [2].

The corticomedullary segment is extremely sensitive to hypoxia; I/R injury promptly results in proximal tubular dysfunction with impaired sodium and water reabsorption. Due to hypoxia and energy depletion, protein degradation occurs leading to the abnormal redistribution of Na^+, K^+-ATPase (NKA) from the basolateral to the apical membrane domain and the cytosol. This redistribution is mitigated by heat shock proteins (HSPs) [3]. HSP72 binds to NKA in a specific and dynamic manner, which is associated with decreased detachment of NKA from the cytoskeleton [4].

We previously demonstrated that NKA stays in the basolateral membrane of renal proximal tubules of females, the enzyme is more stable and is protected from the detrimental effects of I/R injury [5]. Furthermore, we showed that female rats have higher levels of HSP72 than males, and the dynamics of postischaemic HSP72 expression differ between sexes [6]. Our data supports that HSP72 – by stabilizing NKA – plays a relevant role in the sex-dependent vulnerability to renal I/R injury. However, our results and an increasing body of literary data suggest that some of the observed sex-specific differences may be independent from the direct involvement of oestrogen receptors.

We recently described a novel renoprotective signalling mechanism in the kidney. Sigma-1 receptor (S1R) is a highly conserved chaperone protein which has mainly been studied in the central nervous system, but is also expressed in peripheral tissues [7]. We showed that activation of S1R induces the Akt–NOS pathway leading to improved renal perfusion after I/R injury [8]. While the beneficial effects of S1R activation have already been implicated in ischaemic brain [9] and cardiac damage [10], we were the first to describe this protective phenomenon in the kidney.

Beside several drugs and exogenous agonists (e.g. SSRIs, haloperidol, cocaine etc.), S1R can also be activated by various hormone-like ligands such as dehidroepiandrosterone or more importantly 17β-estradiol (E2) [11]. Importantly, E2 and testosterone have been shown to bind to different sites on S1R exerting opposite pharmacological effects. Recent studies indicate that based on its molecular features and pharmacologic regulation S1R might be classified in a unique superfamily of small HSPs [12].

Based on these literary data and our previous results, here we investigated the possible role of sex-specific S1R activation and heat shock response in the renoprotection against severe I/R injury-induced AKI.

Materials and methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

Animals

Experiments were performed on adult female, male and ovariectomized female (Ovx) (200 ± 25 g) Wistar rats. As fluctuating hormone levels can influence the abundance of several proteins, vaginal smears were collected and examined under a light microscope to confirm that all rats were in the proestrus phase when oestrogen levels are high.

All experimental protocols were in accordance with guidelines of the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at Semmelweis University, Budapest, Hungary (PEI/001/1731-9-2015). Rats had free access to standard laboratory diet and tap water.

Experimental protocol

General anaesthesia was induced by intraperitoneal administration of 50 mg/kg pentobarbital sodium (Nembutal, Abbott Laboratories, Budapest, Hungary). Body temperature was maintained at 37 °C on a heating pad throughout anaesthesia. According to the general practice, ovariectomy was performed 7 days before the ischaemic insult to assure full recovery and complete loss of oestrogen [13]. Renal ischaemia was accomplished by cross-clamping the left renal artery and vein for 50 min with an atraumatic vascular clamp. Before the end of ischaemia, the contralateral kidney was removed, the clamp was withdrawn and reperfusion was visually confirmed.

Rats were later re-anaesthetized (50 mg/kg pentobarbital sodium *ip.*), blood samples were collected from the abdominal aorta and kidneys were recovered 2 h (T2) or 24 h (T24) after reperfusion (n = 8/group). Sham-operated rats served as controls (n = 8 per group). Kidney samples were immediately snap-frozen in liquid nitrogen or fixed in 4% buffered formalin (pH 7.4) for further investigation.

Renal functional parameters

Serum creatinine (Cr) and blood urea nitrogen (BUN) were photometrically determined with commercially available kits (Diagnosticum Ltd., Budapest, Hungary) on a Hitachi-712 automated spectrophotometer.

Cell culture and treatment

Human proximal tubular epithelial cell line (HK-2; American Type Culture Collection, Rockville, MD, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) (Life Sciences, Budapest, Hungary) supplemented with 10% foetal calf serum (FCS) 1% L-glutamine and 1% antibiotic, antimycotic solution (100×) containing 10 000 IU/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml Amphotericin B. Cells were incubated in 37 °C, 5% CO_2 and 95% air. There was a 'growth arrest' period of 24 h in serum-free medium before treatment in all experiments.

Cells were treated 24 h prior to harvest as follows: (i) 10 nM E2; (ii) 10 nM E2 + 3 μM S1R antagonist *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE100) (Tocris Bioscience, Bristol, UK). Vehicle-treated cells served as controls ($n = 6$ per group).

Prior to the experiments, the nontoxic dosages of E2 and NE100 were confirmed by methyl-thiazoletetrazolium (MTT) assay (Roche Diagnostics, Mannheim, Germany) (Fig. S1).

Renal histology

Paraffin-embedded, 5-μm kidney sections were stained with periodic acid-Schiff reagent. Sections were coded and examined in a blinded fashion. Glomerular collapse, tubular necrosis, hyalinization, leucocyte infiltration and interstitial lesions were evaluated semi-quantitatively on two fields of ×20 magnification per animal in a double blinded fashion, by two different pathologists as described previously [5].

Fluorescent immunohistochemistry

HK-2 cells were cultured in tissue culture chambers (Sarstedt Kft., Budapest, Hungary). After quick washing the cells were fixed, permeabilized and blocked in one step with Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA, USA). Cells were incubated with specific mouse S1R antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit HSF-1 antibody (1:100; Novus, Cambridge, UK). After repeated washing, chambers were incubated with anti-mouse Alexa Fluor 488 conjugate (1:100; Life Technologies, Budapest, Hungary) and anti-rabbit Alexa Fluor 568 conjugate (1:100; Life Technologies), respectively. Finally, cells were covered with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Budapest, Hungary). Appropriate controls were performed omitting the primary antibody to assure specificity and to avoid autofluorescence. Sections were analysed using an Olympus IX 81 fluorescent microscope (Olympus, Tokio, Japan) with ×100 magnification.

Western blot analysis

All reagents for Western blot were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Total protein was extracted from kidney cortex and HK-2 cells as described previously [8]. Protein concentrations were determined in triplicates by Bradford analysis using bovine serum albumin as standard. Denatured samples (10 or 30 μg) were electrophoretically resolved on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S solution (0.1% w/v in 5% acetic acid) for 3 min, scanned to determine total protein loading and then washed (3 × 1 min) with dH_2O. Membranes were blocked in a buffer containing 20 mM Tris, 150 mM NaCl, 0.5% Tween-20 and 0.1% BSA for 1 h at room temperature (RT).

In the case of cell lysates, membranes were incubated overnight with antibodies specific for human S1R (1:300; Santa Cruz Biotechnology); HSF-1 (1:1000; Santa Cruz Biotechnology); HSP72 (1:9000, donated by Dr. L. László, Eötvös University, Budapest, Hungary); HSP27 (1:1000, HSP27, Ser82) (Novus Biologicals, Cambridge, UK), respectively. After repeated washing, the blots were incubated with the corresponding HRP-conjugated anti-mouse (1:2000; Santa Cruz Biotechnology) and anti-rabbit (1:2000–1:5000; Cell Signaling Technology, Danvers, MA, USA) secondary antibodies.

In the case of kidney tissue samples, membranes were incubated for 1 h at RT with the primary antibodies (1:1000 dilution) specific for S1R (Thermo Fisher Scientific Inc., Waltham, MA, USA), phospho-Akt (pAkt, Ser473) (Cell Signaling Technology), HSF-1 (Santa Cruz Biotechnology), HSP72 (donated by Dr. L. Laszlo, Eotvos Lorand University, Budapest, Hungary), HSP27 (Novus Biologicals) and NKA-α1 subunit (Santa Cruz Biotechnology), respectively. Membranes were then incubated with the corresponding goat anti-mouse or goat anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology) diluted to 1:2000 for 1 h at

RT. Blots were developed with enhanced chemiluminescence detection (AP-Biotech, Buckinghamshire, UK). Computerized densitometry of specific bands was analysed with GEL-PRO ANALYZER 3.2 software.

Integrated optical density (IOD) was factored for internal controls and for Ponceau S staining to correct for any variations in total protein loading [14]. Briefly, the densitometric value of each band was divided by the densitometric value of the entire lane (determined densitometrically using the scan of the Ponceau S-stained membrane). Protein abundance was represented as IOD/Ponceau S/Internal control.

Statistical analysis

Parametrical data are expressed as means + SEM, while nonparametrical data as median ± range. Statistical analyses were performed using GRAPHPAD PRISM Software (version 6.0; GraphPad Software Inc., San Diego, CA, USA). Multiple comparisons and possible interactions were evaluated by one-way ANOVA followed by Bonferroni's *post hoc* test. Histological changes were analysed using the Kruskal-Wallis test followed by multiple pairwise comparisons according to Fisher's test. The criterion for significance was $P < 0.05$ in all experiments.

Results

S1R and HSF-1 are activated by E2 in proximal tubular cells

First, *in vitro* experiments were performed on HK-2 proximal tubular cells to assess the effect of E2 treatment on S1R activation and heat shock response induction. Although S1R protein abundance did not change significantly (Fig. 2a), the receptor's localization was altered upon E2 treatment. S1R showed perinuclear localization in control cells, but was detected everywhere intracellularly and also in the nucleus after E2 treatment, which is consistent with activation of the receptor (Fig. 1).

In parallel, E2 promoted HSF-1 translocation to the nuclei of proximal tubular cells, where it is known to activate the transcription of heat shock response genes (Fig. 1). The addition of S1R antagonist NE100 inhibited the E2-mediated translocation of both S1R and HSF-1 (Fig. 1).

E2 induces HSF-1 and HSP72 production in proximal tubular cells

To test its effect on heat shock response elements proximal tubular cells were treated with 17β-oestradiol. E2 treatment induced significant HSF-1 and HSP72 production; on the other hand, HSP27 remained unchanged at this point (Fig. 2b–d). The addition of selective S1R antagonist NE100 nullified the effect of E2 on all measured proteins confirming the role of the E2-S1R axis in inducing heat shock response (Fig 2b–d).

Postischaemic renal function is better in female rats

The influence of sex hormones on the heat shock pathway was also tested *in vivo*. Both serum creatinine and BUN levels were massively increased at T24 in all groups reflecting the development of AKI ($P < 0.05$, SHAM versus T2; $P < 0.001$, T2 versus T24 versus SHAM and T2, all groups). At T24 Cr, levels were less elevated in female rats (262.3 ± 9.9 μmol/l) compared to both male (361.3 ± 7.8 μmol/l) and Ovx animals (327.5 ± 12.8 μmol/l; $P < 0.001$ resp.), while BUN levels (36.1 ± 1.8 mmol/l) were less elevated compared only to males (50.1 ± 4.1 mmol/l) (Fig. 3).

Structural kidney damage caused by renal I/R injury is milder in females

Renal I/R injury caused extensive tubular necrosis, hyalinization and interstitial lesions as early as at T2, especially in males (SHAM versus T2 $P < 0.05$), (Fig. 4) (for lower magnification see Fig. S2).

Concerning sex differences tubular damage was more prominent in males than in females both at T2 and T24. Furthermore, more severe glomerular collapse and leucocyte infiltration were present in male rats (Table 1).

S1R expression rapidly increases in females after I/R injury

Renal S1R protein levels were the same at baseline in all groups; however, the difference was on the border of significance between SHAM females and males ($P = 0.057$). S1R abundance remained unaltered after I/R injury in males and Ovx, but there was a marked increase in females at T2, which returned to baseline by T24 (Fig. 5a).

Postischaemic pAkt, HSF-1, HSP72, HSP27 and NKA protein levels are higher in females

Renal protein levels of pAkt-HSF-HSP axis were measured to evaluate the signalling mechanism possibly responsible for milder kidney damage in female rats. Baseline pAkt (Ser473; the phosphorylated, active form of Akt) levels were similar in all groups. In females,

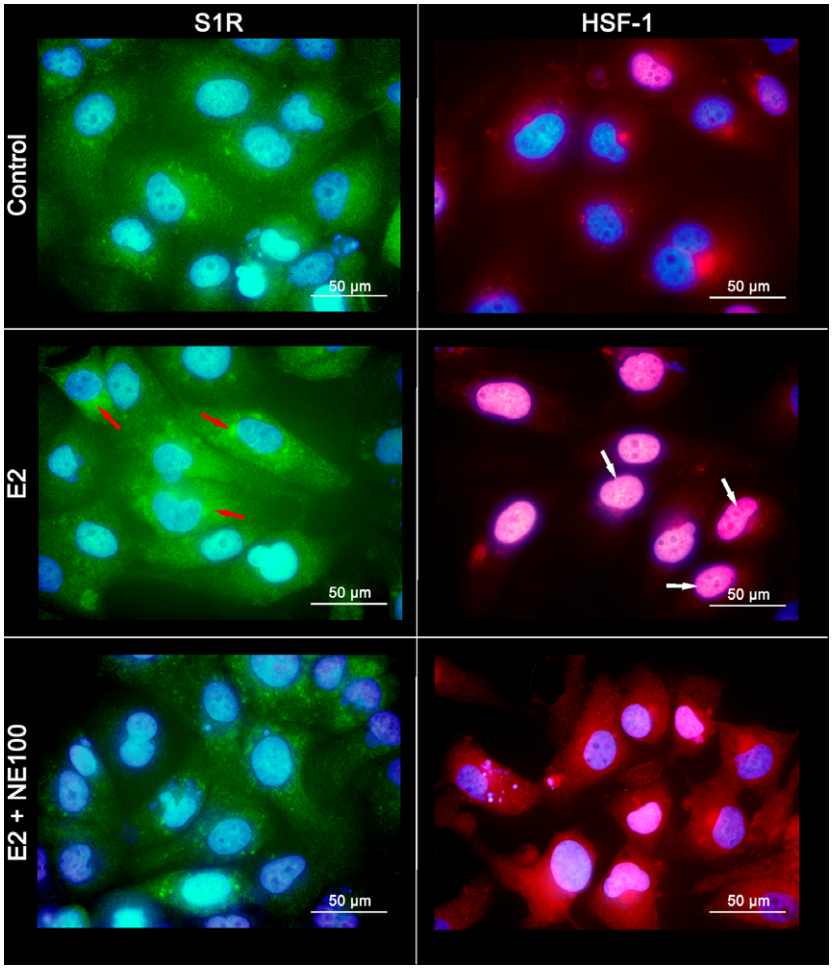

Figure 1 Sigma-1 receptor (S1R) and heat shock factor 1 (HSF-1) are activated by 17β-estradiol (E2) and translocate in proximal tubular cells. Representative images of fluorescent immunohistochemistry staining of Control; 17β-estradiol (E2) and E2 + S1R antagonist *N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE100)-treated HK-2 cells. Red arrows point to S1R in the cytoplasm, white arrows point to HSF-1 in nuclei. Anti-S1R (green); anti-HSF-1 (red) and nuclei (blue); 100× magnification; scale bar = 50 µm.

pAkt protein levels at T2 were almost twofold higher versus males or Ovx ($P < 0.05$). In the latter groups, pAkt increased much slower and reached its maximum only by T24 (Fig. 5b).

HSF-1 was already higher in sham-operated female rats and increased further by T2. At T24 HSF-1 protein levels were further elevated in all groups and were higher in females than in males (Fig. 5c).

Baseline HSP72 protein levels in female rats were considerably higher compared to males or Ovx females. This difference was also apparent as early as at T2 and was even more pronounced at T24 (Fig. 5d).

Elevation of HSP27 protein levels followed different dynamics in time. Baseline HSP27 was higher in females, but decreased to the level of males or Ovx females by T2. By T24 HSP27 levels increased in all groups with higher protein levels in females than in males or Ovx rats (Fig. 5e).

Baseline NKA was higher in sham-operated female rats and increased further by T2 compared both to males or Ovx females. On the other hand, at T24, female NKA levels were still higher than males, but not than Ovx females (Fig. 5f).

Discussion

Sex of the donor and recipient in human kidney transplantation has been associated with diverse short- and long-term graft survival. In a recent paper, Lepeytre *et al.* [16] found that female recipients aged >45 have higher risk of long-term graft failure than their male counterparts. On the other hand, the Analysis of United Network for Organ Sharing data revealed that female sex (both donor's and recipient's) is associated with lower rates of ischaemia-related short-term transplant outcomes, such as delayed graft function; however, the difference is less distinct in patients over

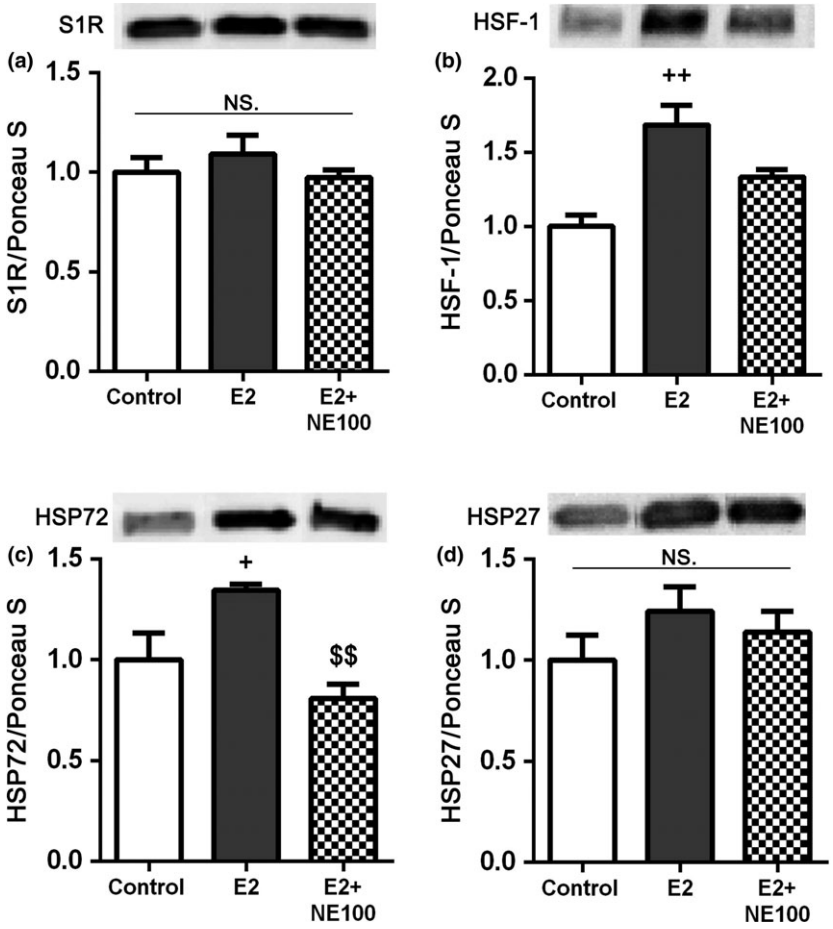

Figure 2 17β-estradiol (E2) induces heat shock factor 1 (HSF-1) and heat shock protein 72 (HSP72) production in proximal tubular cells. (a) Sigma-1 receptor (S1R), (b) heat shock factor 1 (HSF1), (c) heat shock protein 72 (HSP72) and (d) heat shock protein 27 (HSP27) protein levels determined by Western blot in whole cell lysates of Control; 17β-estradiol (E2) and E2 + S1R antagonist *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE100)-treated (24 h) HK-2 cells. $^{+}P < 0.05$ versus Control; $^{++}P < 0.01$ versus Control; $^{\$\$}P < 0.01$ versus E2. White bars represent Control, grey bars E2-treated, chequered bars E2 + NE100-treated cells ($n = 6$ per group). Blots were normalized to Ponceau S staining. Bars indicate means + SEMs. Data were analysed by one-way ANOVA with Bonferroni's multiple comparison test.

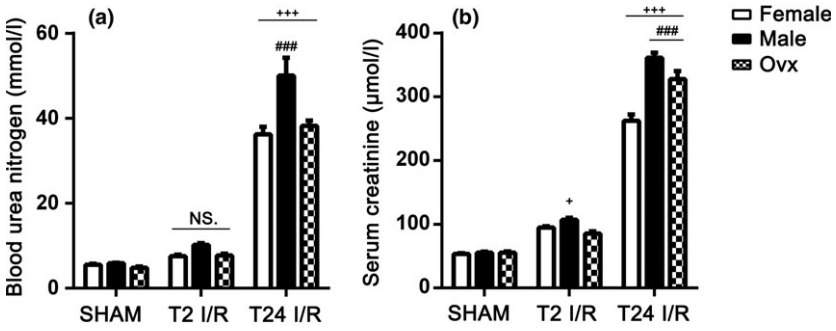

Figure 3 Female sex ameliorates renal functional injury following ischemia/reperfusion (I/R). (a) Blood urea nitrogen (BUN) and (b) serum creatinine of female, male and ovariectomized (Ovx) rats after sham operation (SHAM) or 2 and 24 h (T2 I/R and T24 I/R) after reperfusion. $^{+}P < 0.05$ versus female, male or Ovx SHAM respectively; $^{+++}P < 0.001$ versus female, male or Ovx SHAM respectively; $^{\#\#\#}P < 0.001$ versus T24 I/R female. White bars represent females, black bars males, chequered bars Ovx female rats ($n = 8$ per group). Bars indicate means + SEMs. Data were analysed by one-way ANOVA followed by Bonferroni's *post hoc* test.

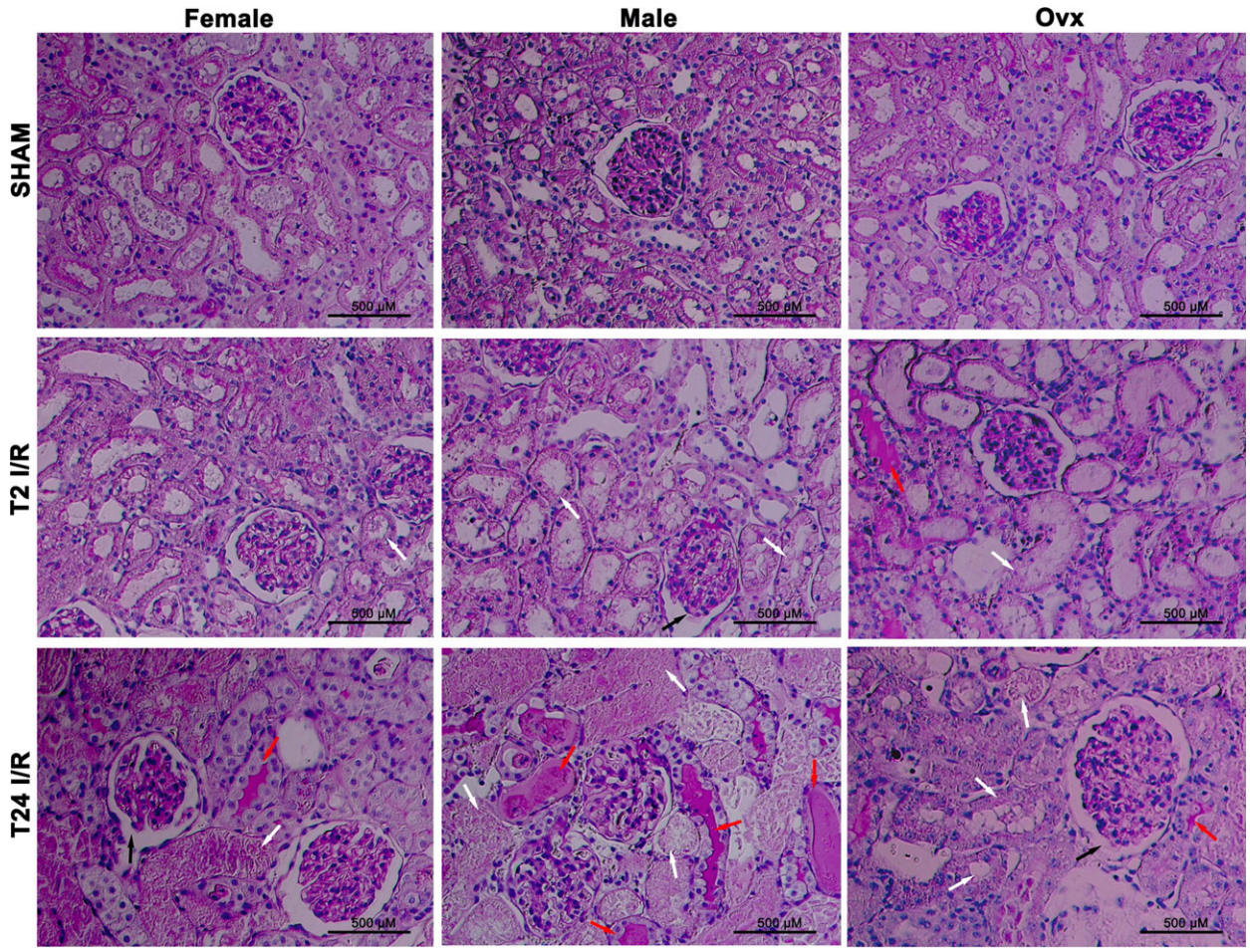

Figure 4 Postischaemic renal lesion is milder in female rats following ischaemia/reperfusion (I/R). Representative periodic acid-Schiff-stained kidney sections show structural damage at 2 (T2) and 24 (T24) hours after reperfusion in female, male and ovariectomized female (Ovx) rats. Black arrows point to glomerular collapse, white arrows indicate necrotic tubules and red arrows show hyaline accumulation. Magnification: ×40, scale bar = 500 μm.

Table 1. Kidney damage following renal ischaemia/reperfusion (I/R).

	Group	Glomerular collapse	Tubular necrosis	Tubular hyalinization	Lymphocyte infiltration	Interstitial lesions
SHAM	Female	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
	Male	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
	Ovx	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
T2 I/R	Female	1 (0–2)+	1 (0–1)+	1 (0–2)+	1 (1–2)+	0 (0–1)
	Male	2 (0–2)+′*	1 (0–2)+	1 (0–1)	1 (0–1)+	1 (0–2)+′*
	Ovx	1 (0–2)+	1 (0–1)+	1 (0–2)+	1 (0–2)+	0 (0–1)
T24 I/R	Female	0 (0–1)	3 (1–4)§	2 (1–2)§	1 (1–2)	1 (0–2)
	Male	2 (2–2)#	3 (2–4)§	2 (2–2)§	2 (1–3)#′§	3 (0–3)#′§
	Ovx	0 (0–2)	3 (1–4)§	2 (1–2)§	1 (1–2)	0 (0–2)

Histopathological changes as glomerular collapse, tubular necrosis, tubular hyalinization, leucocyte infiltration and interstitial lesions were evaluated semi-quantitatively following sham-operation and 2 (T2) and 24 (T24) hours after reperfusion in female, male and ovariectomized female (Ovx) rats. $\pm P < 0.05$ versus SHAM $*P < 0.05$ versus T2 I/R female; $\#P < 0.05$ versus T24 I/R female; $^{\S}P < 0.05$ versus T2 ($n = 8$ per group).

65 years of age [18]. In line with this, other studies described that male sex is an independent predictor of increased incidence and mortality in ischaemia-induced AKI [17].

Several studies investigated the effects of sex steroids on AKI in animal models; however, the role of various sex hormones in the susceptibility to I/R injury is still

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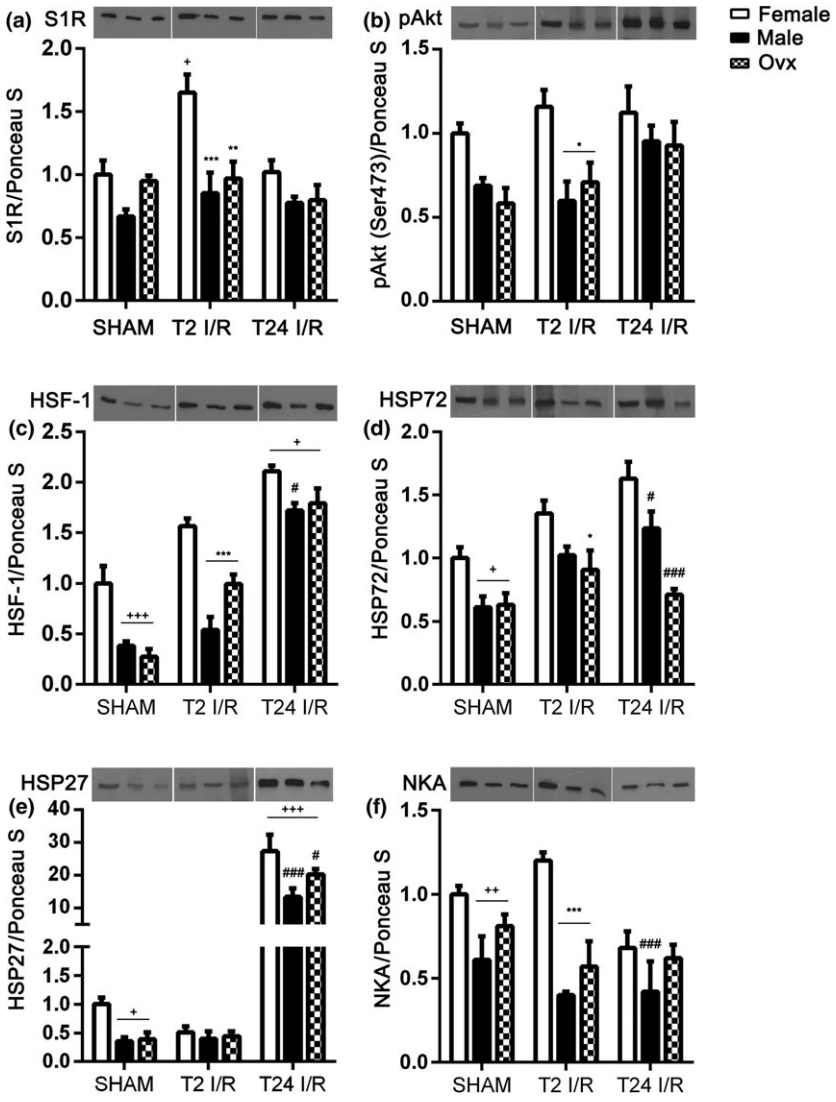

Figure 5 Female sex activates the production of renal Sigma-1 receptor (S1R), phospho-Akt, heat shock factor-1 (HSF-1), heat shock protein 72 (HSP72), heat shock protein 27 (HSP27) and Na^+, K^+-ATPase (NKA) protein following ischaemia/reperfusion (I/R). Renal cortical (a) Sigma-1 receptor (S1R), (b) phospho-Akt (pAKt) (Ser473), (c) heat shock factor-1(HSF-1), (d) heat shock protein 72 (HSP72), (e) heat shock protein 27 (HSP27), and (f) Na^+, K^+-ATPase (NKA) protein levels determined by Western blot at 2 (T2) and 24 (T24) hours after reperfusion in female, male and ovariectomized female (Ovx) rats. $^{+}P < 0.05$ versus female SHAM; $^{++}P < 0.01$ versus female SHAM; $^{+++}P < 0.001$ versus female SHAM; $^{*}P < 0.05$ versus T2 I/R female; $^{**}P < 0.01$ versus T2 I/R female, $^{***}P < 0.001$ versus T2 I/R female; $^{\#}P < 0.05$ versus T24 I/R female; $^{\#\#\#}P < 0.001$ versus T24 I/R female. White bars represent females, black bars Males, chequered bars Ovx female rats ($n = 8$ per group). Blots were normalized to Ponceau S staining. Bars indicate means + SEMs. Data were analysed by one-way ANOVA with Bonferroni's multiple comparison test.

controversial. We and others previously showed that female rats have prolonged survival and improved renal recovery following ischaemia [2,5,6,15]. While a study by Park *et al.* [19] indicated that the presence of testosterone might be more relevant than the absence of E2, our previous data [2] suggested that oestradiol treatment of male rats improves postischaemic survival and kidney function, but castration does not. Further studies demonstrated reduced renal injury after cardiac arrest in E2 receptor knockout female mice directly indicating an E2 receptor-independent protective role of the hormone [20]. Thus, the principal role of sex hormones in sex differences in the mechanism of renal I/R injury is evident, the importance of E2 or its effect on different nonspecific receptors is still not fully elucidated [21].

In the present study we showed that ovariectomy diminishes the protection of female rats. Furthermore, we identified S1R as a possible mediator of protective molecular mechanisms, which could play a part in the renoprotective role of female sex.

Recently, we confirmed the presence of S1R in proximal tubular cells and showed the receptor's translocation upon ligand stimulation [8]. Here we demonstrated that E2 acts directly on S1R and induces the production of heat shock proteins in proximal tubular cells.

Sigma-1 receptors are localized in the mitochondria-associated ER, but can translocate to the cytoplasm upon stimulation [22]. This was confirmed in our *in vitro* experiment where upon E2 treatment S1R translocated from its physiological perinuclear localization to the cytoplasm of proximal tubular cells.

The role of S1R has been demonstrated in models of brain ischaemia and pressure overload-induced cardiac hypertrophy [23,24]. *In vitro* studies also showed that in endothelial cells, S1R facilitates the effects of female sex hormones on endothelin-1 release [25].

The fact that S1R can be activated by E2 taken together with upregulated S1R synthesis in females, but not after ovariectomy suggests a possibly S1R-mediated effect of E2. We propose S1R – a nonspecific receptor of E2 – as a possible mediator of renoprotective heat shock response in females.

Previously, we revealed that S1R activation by ligands induces vasodilative NOS production through Akt phosphorylation [8], thereby improves renal perfusion. Here we showed that S1R and phosphorylated Akt were elevated in female rats compared to males or ovariectomized females 2 h after reperfusion.

The transcription factor HSF-1 is the only one of four HSFs that regulates the expression of HSPs [1,26]. It has recently been demonstrated that Akt is indeed an upstream activator of the HSF-1 chaperone signalling cascade; as the inhibition of Akt using siRNA and small molecule inhibitors prevented HSF-1 activation [27].

Under physiological conditions, HSF-1 is localized in the cytosol in an inactive form. During cellular stress, it is translocated to the nucleus where it activates the transcription of heat shock response genes [28]. Sex hormones have been shown to modulate HSF-1 as it is activated after E2 treatment in male rats. Testosterone treatment on the other hand had no effect on HSF-1 [1]. These data were confirmed in our study, where E2 treatment promoted the translocation and production of HSF-1 in proximal tubular cells. In parallel, we found considerably higher HSF-1 protein levels in female rats than in males or ovariectomized females both at baseline and 2 h after reperfusion suggesting that E2 absence causes decreased HSF-1 response under both normal and pathophysiological circumstances.

HSP72, the inducible form of HSP70, is synthetized under the control of HSF-1. Overexpression of HSP72 protects cells and tissues against ischaemic injury in various organs [29]. Here we showed that HSP72 levels in ovariectomized females were similar to that of males underlining the possible role of E2. This is in line with the findings of Voss *et al.* [30] who reported that ovariectomy reduces HSP72 levels to that of males in the heart, which can be prevented by E2 supplementation.

Disruption of the actin cytoskeleton in proximal tubular epithelial cells is a prominent feature of ischaemic injury and is known to be influenced by sex hormones [31,32]. This process leads to the translocation of NKA from the basolateral membrane of tubular cells making it dysfunctional [6,31]. Both HSP72 and HSP27 (small heat shock protein) protect cells from injury not only by their chaperone activity but also by stabilizing the actin cytoskeleton and thus HSPs have a pivotal role in preserving renal function. HSP72 levels were highest in females at T2 and could be responsible for stabilizing the cytoskeleton – and thus NKA at its physiological location – at this early time point. In line with previous findings in the present study, HSP27 accumulated in a more prolonged manner than HSF-1 or HSP72, amounting to significant quantities 24 h after reperfusion [33]. Importantly female rats had significantly higher HSP27 levels than males or ovariectomized females.

In summary, we confirmed the role of sex hormones in superior outcomes in females after renal I/R injury and propose a S1R-mediated molecular pathway which could contribute to the renoprotection females enjoy. Our results indicate that activation of S1R by E2 is protective by enhancing the heat shock response in the kidney.

Authorship

AH: performed research, wrote the paper. ZA: performed research, wrote the paper. AV-S: performed research. LL: performed research. DBB: performed research. ES: performed research. LI: analyzed data. JH: performed research. NFB: performed research. LW: wrote the paper. AV: designed study. AJS: designed study. AF: designed study, wrote the paper.

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Conflict of interest

The authors have declared no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Cell viability assay of HK-2 cells after 17β-estradiol (E2) or NE100 treatment.

Figure S2. Postischemic renal lesion is milder in female rats following ischemia/reperfusion (I/R).

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